Genetic Diversity in Chickpea based on Morphometric and Molecular Markers

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By

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In the Name of Allah, Most

Gracious, Most Merciful

Dedicated To

My Loving Parents

And Dear teachers

DECLARATION

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LIST OF ACRONYMS

%	Percent
mg	micro gram
µl	micro liter
µm	micrometer
0C	Degrees Celsius (Centigrade)
Mbp	Million base pair
Pg	Pico gram
SDS-PAGE	Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeat
RFLP	Restriction Fragment Length Polymorphism
AFLP	Amplified Fragment Length Polymorphism
SNP	Sequence Nucleotide Polymorphism
PCR	Polymerase Chain Reaction
MAS	Marker Assisted Selection
HI	Harvest Index
CV	Coefficient of Variation
USDA	United State Department of Agriculture
BPB	Bromo-Phenol Blue

UPGMA	Unweighted Pair –Group Method Arithmetic Average
Tris-HCl	Tris Hydrochloric acid
Nacl/ Mgcl2	Sodium Chloride/ Magnesium chloride
PGRP	Plant Genetic Resource Programme
RCBD	Randomized Complete Block Design
STMS	Sequence Tagged Microsattelite Sequence
Psi	Pressure per square inch
PH	Proportionate Hydrogen ions
NARC	National Agriculture Research Centre
ICRISAT	International Crop Research Institute for Semi Arid Tropics
ISSR	Inter Simple Sequence Repeat
ICARDA	International Centre for Agriculture Research in Dry Areas
ha	hectare
dd	double distilled
FOC	*Fusarium oxysporium ciceris*
S.E	Standard Error
Std.dev.	Standard deviation
tEE	Total Environmental Error
M. wt	Marker weight
bp	base pair

USA	United State of America
Acc.	Accession
HR	Highly Resistant
R	Resistant
SR	Susceptible at Reproductive to pods maturity stage
SS	Susceptible at Seedling stage
CTAB	Cetyl Trimethyl Ammonium Bromide
EDTA	Ethylen –Diamin- Tetra- Acetate
PVP	Poly Vinyl Pyrrolidone
rpm	revolution per minute
TBE	Tris Borate EDTA
dNTP	Deoxy Nucleic acid Tri-Phosphate
Kb	Kilo base
P- value	Pearson Correlation
KDa	Kilo Dalton

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Zakia Ahmad

ABSTRACT

The exploration of genetically variable accessions is the key source of germplasm conservation and potential breeding material for the future. The more diverse group of cultivars can provide an ample opportunity to breeders for releasing new and superior varieties, considering their quality traits for direct commercial utilization. In advanced research studies the polymerase chain reaction (PCR) based molecular markers have a great contribution in genome analysis and marker-assisted selection. In this study, the genetic diversity of *Cicer arietinum* L. twenty four indigenous and forty six exotic accessions were assessed, obtained from plant genetic resource institute (PGRI), national agriculture research centre, Islamabad, Pakistan. These accessions were planted under field conditions at research area of University of Malakand, Chakdara, Khyber Pakhtunkhwa. The genetic diversity among seventy chickpea indigenous and exotic accessions was estimated using morphological, biochemical; sodium dodecyle sulphate polyachryl amide gel elecctrophoresis (SDS-PAGE) and molecular markers; random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers.

Based on qualitative and quantitative morphological traits, the average coefficient of variation (%) was calculated 44.8% and 56.8% respectively with significant correlation among yield traits. The analysis revealed that the accessions 1898, 2819, 3022, 3037, 3040, 3043, 3054, 3059 and 3063 were best in performance with a total of 12% environmental error. The statistical analysis showed that 100 seeds weight was significantly correlated with seed size quantitatively. The majority of accessions of USA origin were observed with maximum100 seed weight (30-57gm) and medium to large (7.2- 9.9mm) size seeds including one of the Pakistani accession 2562 also with large size seeds. The wilt incidence (%) was observed to be comparatively higher (30 - 42.85%) at both growth stages in field screening of the germplasm than that of greenhouse conditions; reduced up to 8.57% at seedling stage and 24.28% at reproductive stage. The *t-test* however, indicated that chickpea both from indigenous and exotic origin showed a significant variation at alpha ≤ 0.050 at seedling and reproductive stage.

The cluster analysis based on protein data indicated 50% genetic diversity among the accessions. The clustering pattern did not reveal any grouping that could be attributed to either the geographic distribution or the field performance. For molecular characterization of

germplasm twenty random amplified polymorphic DNA (RAPD) and twenty simple sequence repeat (SSR) polymerase chain reaction (PCR) based markers were screened for estimation of genetic variability. In the markers, five random amplified polymorphic DNA (RAPD) and fifteen simple sequence repeat (SSR) were polymorphic and showed significant level of coefficient of variation. The data of molecular markers were scored by the presence (1) and absence (0) of allele and subjected to statistical analysis. The analysis was based on coefficient of molecular similarity using un-weighted pairs group mean average (UPGMA) resulted in 37% and 55% genetic diversity among the total germplasm using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers respectively.

For marker trait association analysis, twenty random amplified polymorphic DNA (RAPD) and twenty simple sequence repeat (SSR) makers were utilized to find correlation of markers with yield contributing components and chickpea *Fusarium* wilt resistant genes. None of the random amplified polymorphic DNA (RAPD) primers were linked to seed size and seed weight while, simple sequence repeat (SSR) markers TA72 and TA130 showed association at linkage distance 0.4 with seed weight and seed size. Based on which the high yielding accessions among chickpea germplasm were identified. Hence, the association of these makers is helpful for the plant breeders to select lines on the basis of yield contributing traits. Among the total used random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) primers, TA194 (SSR marker) was linked to the disease response with 85% probability level. This association or correlation of the marker was reconfirmed by receiver operating characteristic curve (ROC). Hence, the use of the sorted wilt resistant genotypes through simple sequence repeat (SSR) marker TA194 can make available ample prospect in marker assisted breeding for yield improvement of chickpea in Pakistan.

INTRODUCTION

Food legumes play a vital role in terms of nutritional value all over the world, as it is a source of protein and minerals. Agronomically legumes serve as rotation crop with cereals, reducing pathogens and enhancing the supply of nitrogen to the soil (Sitou and Mywish, 2011). The legumes belong to family Leguminosae or Fabaceae with more than 20,000 species. These are considered second to cereal crops mainly on the basis of production (Graham and Vance, 2003).

Chickpea (*Cicer arietinum* L.) is a well known member of the family Fabaceae, included in its subfamily Papilionaceae (Nasir and Ali, 1972). Chickpea is given worldwide different common names which are Bengal gram, Chickpea or Garbanzo, Chana, Hommes or Hamaz, Nohud, Lablabi and Shimbra in India, Europe, Pakistan, Arabic countries, Turkey and Ethiopia respectively. The genus comprises one cultivated species, *i.e.*, *Cicer arietinum* L. and 42 wild species (Vavilov, 1951).

The Taxonomic characteristics of the species revealed that the stem is erect, branched and shrubby, 0.3-1m tall, glandular pubescent, color is olive, bluish green or dark green. Tap-root system with 3-4 well-defined rows of lateral roots; Primary and secondary roots usually develop large lobed nodules containing rhizobia for fixing atmospheric nitrogen. The main stem produces quadrangular, ribbed branches and sometime profusely branched at various levels (Duke, 1981). These may be erect, semi-erect or prostrate. Leaves with 3-10 pairs of leaflets which are ovate or elliptic, 0.7-3.0cm wide; margin serrate, however, some exceptions with a highly dissected compound leaf or with simple leaf lamina also occur, leaf apex aristate or acuminate with cuneate base; stipules if present are 2-5 toothed. The inflorescence is axillary or solitary with 0.5-2.9cm long peduncles, pedicels 0.5-1.3cm in length, flower bracteate; corolla purplish, white or pink purplish; stamens are in diadelphous (9-1) condition surrounding sessile ovary (Duke, 1981; Van der Maesen, 1987; Cubero, 1987). Pod is 1.5-2.5 cm long usually with three seeds and the surface of the pod is glandular-pubescent; seed coat wrinkled or smooth with a median groove and anterior beak; germination is observed as cryptocotylar

(Duke, 1981; Van der Maesen, 1987; Cubero, 1987). It is a self-pollinating crop and rarely cross pollinated (Smithson *et al*., 1985; Singh, 1987). It is cool-weather, rain fed and dry climate crop (Rao *et al*., 2010). In Pakistan it is sown in the middle of September to November or rarely later and is matured in 3-6 months.

It has been reported that chickpea origin is from south-eastern Turkey (Ladizinsky, 1975). However, it is worthwhile to mention that the main chickpea producing regions of the world are Canada, America, Australia, Mexico, India, Pakistan, Turkey, Iran, Ethiopia and Myanmar (Nawroz and Hero, 2011). According to one of the report submitted by FOASTAT in a year 2008, it was grown on an area of about 10.7 million hectares with 8.2 million tons annual production. Pakistan is major chickpea grower country, where it is cultivated on about one million hectares with a total production of 760 thousand tons (GOP, 2009). The contribution of chickpea among pulses production is up to 70% which covered 82%, 9%, 8% and 1% area of Punjab, Khyber Pakhtunkhwa, Sindh and Baluchistan respectively (Ansar *et al*., 2010). Although it is grown on large area, but the main reasons of its very low yield and production are either biotic/ abiotic stresses, selection strategies for development of desirable traits cultivars, inaccessibility of disease free seeds to the farmers and poor labour management (Pankaj, *et al.*, 2001; Upadhyaya *et al*., 2001; Sharif, 2004; Hassanuzzaman *et al.*, 2007; Cani and Toker, 2009; GOP, 2009; Gaur *et al.*, 2010).

Among various environmental constraints, one of the limiting factors that directly affect the yield and cause 10-90% loss to the crop (Jimenez-Diaz *et al.*, 1989) is the fungal disease caused by *Fusarium oxysporum* sp. *ciceris* (Schlechtends) which causes wilting. At least 8 races of this fungus have been reported, out of which 6 are more virulent causing wilt disease (Jimenez-Diaz *et al.*, 1993; Kelly *et al*., 1994), giving no information on existence of races in Pakistan (Mahmood *et al*., 2011). Chickpea wilt is gradually prevailing in Pakistan as a result of the increased drought condition since for the last few years. Therefore, the issue needs great attention to enhance the yield (Lines *et al.*, 2008). The disease is soil or seed born, which is difficult to control by the use of chemicals or fungicides (Farhat *et al*., 2010). To overcome this serious problem, the use

of wilt resistant cultivars is the best and cheapest choice for breeders to adopt (Sharma *et al*., 2005; Mahmood *et al*., 2011).

Seed size which has a direct relationship with seed weight in chickpea is one of the important growth parameter to consider for increasing the yield (Narayanan *et al*., 1981; Vadivelu and Ramakrishnan, 1983; Dahiya *et al*., 1985; Upadhyaya *et al*., 2006). Therefore, for food security of the day to day increasing population it is imperative to enhance the production rate of the crop up to the actual demand by considering the important growth parameters of chickpea crops.

Regarding their nutritional status chickpea contains protein (12.4-31.5%), water soluble vitamins, carbohydrates (48.1-68%), starch (41-50%), 4.8% oil, fat (6.2%), 3% ash, 3.1% fiber, 0.3% phosphorus and 0.2% calcium (Yousefiara *et aI*., 2008; Huda *et al*., 2003).

For optimizing health issues and prevention of diseases, the American diabetes association, heart association and cancer society suggested that legumes are key foods in daily life. Medicinally chickpea is used for regulating the level of cholesterol, triglycerides, sugar and insulin secretion. It also used for the treatment of cholera, diarrhea, bronchitis, constipation, dyspepsia, catarrh, snake biting, cutamenia, warts, colon cancer and cardiovascular disorders. The Seeds are antibilious and antioxidant (Duke, 1981). Chickpeas added a significant amount of nitrogen in the soil to improve its health and fertility (ICRISAT, 2005).

Keeping in view the high economic importance of cultivated chickpea, its 931 Mbp small sized genome (Nawroz and Hero, 2010) and 3 to 6 months life cycle make it an important species for genomic research. The yield can be increased by using the germplasm for new genes, also reported by many workers (Radhika *et al*., 2007; Sefera *et al*., 2011; Thudi *et al*., 2011). The polygenes which are concerned with the inheritance of agronomically important quantitative traits, individually less effective to express themselves in phenotype and difficult to identify. This leads to revise and further evaluate the germplasm to measure the level of genetic diversity it contains and to ensure its maintainance in a more effective and efficient way (Ghafoor *et al*., 2000; Nisar *et al*.,

2008). It is necessary to use the broader range of genetic diversity to meet the needs of more food (Karoaz and Zencirci, 2005). Assessment of the extent of genetic diversity is fundamental in chickpea breeding and genetic resource conservation for selection of parents to produce hybrids (Dwevedi and Gaibriyal, 2009; Agrawal and Srivastava, 2010). Therefore, to assess the genetic variability within different accessions or natural populations the use of DNA based molecular markers is the most authentic tool and stable macromolecules, free from most of the environmental influences (Vural and Akein, 2010; Datta *et al*., 2010; Mahmood *et al*., 2011). To improve the quantitative traits large number of genotypes are required to evaluate following classical breeding programs which are unaffordable, more difficult and under the influence of environmental stresses. This needs an alternative program to replace the traditional procedures by marker assisted selection (Allahverdipoor *et al.*, 2011). Thus the selection and inheritance of the desirable traits is now becoming possible with the advancement of marker assisted selection (MAS), associated with the expression of specific genomic region for the selection of certain desirable traits. It provides a beneficial source to exploit the potentiality of genes against agronomic traits and to determine the genetic diversity among local and exotic accessions (Choudhry *et al*., 2008). In advance research studies the polymerase chain reaction (PCR) based molecular markers have a great contribution in genome analysis and marker-assisted selection (Datta *et al.*, 2010). Recently, the technology of molecular markers has been greatly developed for plant breeding. In this way simple sequence repeat (SSR) techniques can be used for direct selection of desirable traits, when linked them with traits of interest (Edwards and Mogg, 2001).

Estimation of genetic diversity based on morphometric and biochemical analysis using sodium dodecyle sulphate polyacryl amide gel electrophoresis (SDS-PAGE) (Mennella *et al*., 1999, Netra and Prasad 2007, Nisar *et al*., 2007, Nisar *et al*., 2011) and, molecular characterization with the help of random amplified polymorphic DNA (RAPD) markers (Talebi *et al*., 2008; Ahmad *et al*., 2010; Mahmood *et al*., 2011); simple sequence repeat (SSR) markers (Sun *et al*., 1998) has been carried out in the present investigation to verify the existence of correlation between genetic and morphological variability among the indigenous and exotic accessions of both desi and kabuli chickpea

and to select promising lines regarding their stability in performance. In Pakistan no true work has been found to increase the yield of the crop so far due to the presence of wide gap between its potential and real yield attributed by different constraints (Pankaj, *et al*., 2001 and Sharif, 2004). Unfortunately in traditional farming system the farmers still use primitive chickpea cultivars due to the unavailability of the attainments of chickpea upgrading research programs to increase the yield at homestead level. However for a substantial increase in chickpea production which is the requirement of developing countries like Pakistan to overcome food problems, there is a needed to adopt the use of quality seeds with allied scientific technologies by the chickpea growers. Another causal agent of low production of the crop is very less attention, has been observed by the private sector to upgrade the outcome of this highly nutritious and low cost crop (Gaur *et al*., 2011). In spite of all the described problems confronting chickpea production in the country, the yield can be stabilized and improved by the development of suitable chickpea cultivars adaptable for all sorts of environments (Bakhsh *et al.*, 2011).

The present study is the very first attempt in Pakistan to assess and compare the genetic variability among the indigenous and exotic accessions of chickpea by applying all of the criteria *viz*., morphological traits, biochemical methods or molecular markers for estimation of genetic diversity to select promising lines in terms of high and stable yield and resistant to environmental stresses for future chickpea breeding programs. All the techniques for estimation of genetic diversity have their own implication and validity; none of them is superior, but have weightage on the reproducibility and character stability. In addition to observe the inference of seed size upon seed weight and ultimately on yield direct selection of more stable genotypes were used for field screening and microsatellite markers reported in different studies. Thus the study is proposed to check the level of genetic correlation of seed size and seed weight and it was further hypothesized that is there any sort of correlation of the quantitative traits (yield contributing traits) with the molecular markers: random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) makers.

The virulent races of the pathogens need continuous characterization for screening of germplasm because of constantly changing their nature after some time from resistant to susceptible (Porta-Puglia, 1989; Jamil *et al*., 1995; Haware & Nene, 1982; Jimenez-Diaz *et al*., 1989; Jamil *et al.,* 2010). Moreover, the conventional pathotyping techniques are no more valid now for reliable evaluation and identification of wilt causing fungal pathogens (Jamil *et al*., 2000). Therefore, the identification and isolation of the resistant and susceptible lines through a set of random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers linkage analysis was also undertaken in the present investigation for future resistance gene pyramiding and to enhance resistant germplasm resources for increasing yield of chickpea in Pakistan.

MAIN OBJECTIVES OF THE STUDY

The study was conducted to find the genetic variability in *Cicer arietinum* L. using morphometric, biochemical, disease screening and molecular markers. The main objectives were:

1. The identification/ selection of elite genotypes with superior morphological and agronomic traits.
2. To determine the correlation among quantitative traits.
3. To evaluate and screen chickpea germplasm in a field against *Fusarium* wilt in order to select resistant lines.
4. Marker assisted selection (MAS) for seed weight and seed size.
5. Marker assisted selection (MAS) for *Fusarium oxysporum* wilt resistance.
6. Investigation of genetic diversity in chickpea germplasm on the basis of sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and polymerase chain reaction (PCR) based random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers.

REVIEW OF LITERATURE

2.1 Chickpea cultivation in Pakistan

Two types of chickpea, small, dark-seeded desi type of Indian origin and large, light-seeded kabuli type of Mediterranean origin (Khan *et al.*, 2012*)* have been reported to be produced globally. The worldwide average production of desi type is 75% and that of kabuli type is about 25% as estimated by Wang (2004). Morphologically, the flowers of desi types are pink while those of kabuli types are white in color. The best time for its cultivation is the end of the rainy season which facilitated farmers for double cropping practice and increase the source of income with increasing productivity (Kassie *et al*., 2009). Chickpea is an important component of agriculture particularly grown under irrigated system, rice based system and Rain fed system in Pakistan constitute 1%, 11% and 88% of the total area respectively (Malik, 1994). Chickpea is mainly grown in moist soil after harvesting of rice both in Sindh and Baluchistan and is produced in barani areas of Khyber Pakhtunkhwa and Punjab. The kabuli type chickpea is mostly cultivated in temperate and desi type in semi-arid tropical regions (Muehlbauer and Singh, 1987; Malhotra *et al*., 1987). In Punjab it contributes 90% of production frequently cultivated in Layyah, Jhang, Jhelum, Chakwal, Attock, Bhakhar, Khushab and Mianwali districts (Haqqani *et al*., 2000). Seeds are sown in September-January in Pakistan and India (Smithson *et aI*., 1985). In addition the Crop takes 3-7 months for maturation and harvested at maturity or slightly earlier. It is a "day-neutral", quantitative long-day plant and flowers in each photoperiod (Smithson *et aI*., 1985).

Among the important grain legumes, chickpea (*Cicer arietinum* L.) is considered as third staple food after pea and bean (Sidramappa *et al*., 2010). It is cultivated mainly in South Asian countries with significant nutritional and cultural value (Nawroz and Hero, 2011).

2.2 Germplasm resources of chickpea

The initial collection of chickpea germplasm resources was assembled by

Regional pulses improvement project (RPIP), a joint project of Indian IARI, USDA in U.S and Karaj Agricultural University of Iran. Recently, it is maintained in India at ICRISAT and in Syria at ICARDA (Upadhaya *et al*., 2008).

Evaluation and characterization of germplasm for quantitative and qualitative traits of a plant species and its utilization has received attention from plant breeders because of increased recognition of germplasm reserves and its importance in proper genotype identification (Virmani *et al*., 1983; Ghafoor *et al*., 1992; Bakhsh *et al*. 1992; Pezzotti *et al*., 1994; Rabbani *et al*., 1998). The proper identification and utilization of available germplasm is useful not only in selection of core collection but can also provide basic information about genetic diversity and plays a key role in successful breeding programs (Ranganayaki *et al*., 2001).

In the development of genetic maps using molecular markers mostly quantitative traits are being selected by breeders in crop improvement programs. Thus, the use of DNA markers in marker assisted selection is considered a modern tool in Agriculture to construct complete genome map for improving Plant breeding strategies (Simon and Muehlbauer, 1997). Marker assisted selection has proved and identified the effect of polygenes together with environment as well as small effect of individual gene upon quantitative trait loci (QTLs) in phenotype (Chaudhry *et al*., 2008).

The investigation of genetic diversity is extremely important for effective utilization of germplasm resources (Smith and Smith, 1989). Similarly, the distribution of diversity in phenotypic and genotypic variations among local and exotic lines of various crops including *Cicer arietinum* L. has been examined by many researchers. Therefore, collection of germplasm material from diverse geographical regions revealed high genetic variability which is beneficial for increasing the size of gene pool and insurance of co-adapted genes conservation (Brown, 1978; Frankel and Soule, 1981; Frankel, 1984; Beuselinch and Steiner, 1992; Frankel *et al*., 1995). Moreover, Simmonds, 1979 developed an idea that genetically heterogeneous lines are far better for stable yield than that of homogeneous population. It would be necessary for future breeding to broaden the range of genetic diversity in plants to overcome the world's food problems (Karaoz and

Zencirci, 2005; Farshadfar and Farshadfar, 2008).

2.3 Morphological characterization of chickpea germplasm

Morphological characterization is considered as the first step to describe and classify the available germplasm (Smith, 1989). Tremendous variations for economically important quantitative and qualitative traits, including seed weight and size, growth duration, yield and biomass, plant height, shape and color of grain, flower color, podding, color of seed coat, earliness, resistance to diseases and other quality traits need to be documented and recorded which help breeders to release improved cultivars and varieties (Collard *et al*., 2007; Dasgupta *et al*., 1987; Singh and Ocampo, 1997). The main objective of most breeding programs is to increase the yield (Singh and Auckland, 1975; Byth *et al*., 1980; Lal and Tomer, 1980). The importance of germplasm can be determined by observing the individual accessions agronomical and morphological traits along with their resistance potentiality against environmental stresses. Similarly the economic value of a population has direct relationship with its nutritional qualities and agronomic performance (Piergiovarrni *et al*., 2000). Earlier workers including Sharma *et al*., (1969); Sandhu and Singh, (1972); Gupta *et al*., (1972); Katiyar *et al*., (1970) and Wadud and Yaqoob, (1989) reported that grain yield has a positive relationship with 100 seed weight, number of branches and number of pods. However, Wadud & Yaqoob, (1988a, b) reported a negative relationship between grain yield and plant height. The high pod bearing bold seeded genotype may produce high grain yield (Tomer *et al*., 1973 and Malik *et al*., 1983). Soomro and Larik, (1981) observed non-significant negative correlation between plant height and grain yield.

Several workers reported highly significant positive correlation among secondary branches, pods per plant and yield (Bakhsh *et al*., 1991; Balyan and Singh, 1986; Sarwar *et al*., 1982), and recommended such traits as selection criteria for Chickpea breeding. Singh *et aI*., (1978) studied selection index based on the pods' number, primary and secondary branches and was recommended to improve yield in Chickpea. Smithson *et al*., (1985), Farshadfar and Farshadfar, (2008), Tuba and Sakar, (2010) recorded that the pod number was positive significantly correlated with per plant seed yield in more than 60

cases, with "r" value ranging from 0.28- 0.95%, and significant negative correlation between these characters was never published in Chickpea. Zahoor and Rabbani, (1992), studied morphological traits *viz*, days to maturity, pods per branch, pod length, seed number, 100 seed weight and different attributes for correlation of yield. Furthermore, Ghafoor *et al.*, (1993a, b) added that the biomass is significantly associated with pods and branches per plant; therefore, the biomass is useful for the selection of genotypes for more number of pods and grain yield. However, Katiyar, (1979) observed positive association of pod per plant which was significantly negative with days to maturity.

Iqbal *et al*., (2003) used the selected quantitative traits; height, branches, pods/ plant, pod width, chlorophyll contents, leaf area, root length, root weight, number of locules, biological yield, seed/ pods, grain yield, 100 seed weight, seed set percentage, seed width seed length and harvest index for selection of promising lines of chickpea. Dasgupta, (2003) evaluated genetic diversity among 23 advanced lines and 2 control cultivars of chickpea and found these lines significantly varied for plant height, 50% flowering, days to maturity, number of pods and branches, pod area, seeds in each pod, seed weight 100 and harvest index.

Traditionally, diversity is assessed by measuring variation in phenotypic traits, which are of direct interest to users (Farshadfar and Farshadfar, 2008). The Grain yield and many related traits correlation coefficient showed linear relationship and path analysis would elucidate direct and indirect relationship among these traits, hence on the basis of that the breeder could select the most effective traits to release varieties (Ulukan *et al*., 2003, Yucel *et al*., 2006). According to Saleem *et al*., (2002), Noor *et al*., (2003), Toker, (2004) the pod number and 100 seed weight were the most desirable traits for chickpea improvement. Sensitivity of chickpea to greater concentration of salts in a soil also has an adverse effect on germination, yield and biomass (Ahmad *et al*., 2005). The increase in number of resistant or tolerant genotypes by crossing wild species with cultivated species of *Cicer* is the aim of breeders (Singh *et al*., 2008; Malik *et al*., 2011). Abbo *et al*., (2005) used advanced technique in order to study the relationship of seed weight and concentration of beta-carotene and lutein by using high performance liquid

chromatography after crossing Israeli cultivars with wild *Cicer reticulatum* Ladiz.

The phenotypes association with genotypes is considered usually more common in breeding strategies, as well as in the history of plant domestication carried out through the selection of better plants have shown the correlation between genotypes and morphological traits is relatively low (Bar-Hen *et al*., 1995; Kwon *et al*., 2005; Lefebvre *et al*., 2001; Tommasini *et al*., 2003). Kozak *et al*. (2011) concluded that in diverse environments the phenotypes also varied from each other. However, Khan *et al*. (2008), Dwevedi and Gabriyal, (2009) worked on the same subject to assess the genetic variability among chickpea genotypes by using morphometric and economically important quantitative traits. They concluded maximum phenotypic and genotypic coefficient of variation (PCV, GCV) and heritability in grain yield, 100 seed weight and number of pods. A lot of data have been published regarding similar information, however, the data on trait *i.e.* total biomass which also has significant positive correlation with 100 seed weight and grain yield in chickpea are scanty. Therefore, it is necessary to explore and identify unreported chickpea cultivars with all the valuable quantitative traits of interest for future breeding strategies.

2.4 Biochemical evaluation of chickpea germplasm

Plant germplasms are the genetic resource materials for the development of new and superior cultivars. Germplasm may exist in the form of seeds, leaf, pollen, stem, or cultured cells to develop plants which are identical to their parents. Germplasm characterization and proper evaluation is important for crop improvement (Masood *et al*., 2004).

The data on morphological, agronomic and physiological traits are usually used to estimate magnitude of genetic diversity present in the germplasm, which is not enough for effective management of plant genetic resources. However, due to the effect of environmental factors upon the expression of certain traits may not produce an accurate indication regarding genetic diversity of germplasm (Jomova *et al*., 2009). Molecular and biochemical markers are thus considered as the best option in evaluation of genetic

diversity both in germplasm accessions and natural populations which is also important for evolutionary and phylogenetic studies (Dakir *et al*., 2002).

Among the biochemical techniques, SDS-PAGE or sodium dodecyl sulphate polyacrylamide gel electrophoresis is a simple and economical and extensively used technique for describing the seed protein diversity of crop germplasm (Cook, 1995; Das and Mukarjee, 1995; Fufa *et al*., 2005; Iqbal *et al*., 2005). Furthermore, seed proteins are used as genetic markers in the study of genetic variation because these are the primary products of structural genes and any change in the coding sequence of a gene generally reflects the corresponding change in the primary structure of protein (Srivalli *et al*., 1999). The protein profiling pattern and application of genetic markers always provided effective information to determine several crops evolutionary and taxonomic aspects (Khan, 1990; Murphy *et al*., 1990; Das and Mukarjee, 1995; Ghafoor *et al*., 2002). Analyses of proteins are useful for characterization and identification of diversity in different cultivars as well as to find their phylogenetic relationship (Nisar *et al*., 2007).

2.5 Molecular analysis through PCR based DNA markers

The morphological and biochemical markers are usually under the influence of growth practices and environmental factors, whereas, DNA based markers described genome sequence composition which make possible the detection of information regarding genetic diversity (Iruela *et al*., 2002). In molecular biology DNA based markers have been recognized to detect the genetic variability and phylogeny in different accessions or species of natural population (Kaundun and Park, 2002). Assessment of the level of genetic variability in chickpea germplasm is a key point for chickpea improvement and conservation of genetic resources, also useful in hybrids formation (Talebi *et al*., 2008). Cultivated *Cicer arietinum* is self-pollinated crop shown less intra and inter-population variability (Crawford 1990). In this connection to overcome the problem of a low level of polymorphism in cultivated chickpea PCR-based techniques are prerequisite to revolutionized (Varshney *et al*., 2007). The conservativeness of flanking regions induced by microsatellite in *Cicer* L. creates the possibility to use already available DNA based markers (Choumane *et al*. 2000; Sethy *et al*. 2006).

In plant improvement programmes mostly the quantitative traits are considered by the breeders which are usually controlled by polygenes and influenced by environment (Choudhary *et al*., 2008). Molecular markers are now used for selection of superior and resistant parents with desirable traits than the existing ones through marker assisted selection (MAS). DNA fingerprinting for cultivar or varietal identification facilitating cloning of genes, genome organization and marker assisted selection of morphological characteristics and conservation programs (Gaur *et al*., 2011). Similarly, the increased use of molecular markers in recent years is due to polymerase chain reaction (PCR) based markers used in the assessment of genetic variability in crop plants (Datta and Lal, 2011). The morphometrics and molecular markers have shown different criteria for the determination of genetic diversity among cultivars (Upadhaya *et al*., 2007; Sharma *et al*., 1995). In this connection a number of molecular markers (AFLP, RAPD, RFLP, SSR, SNP and CAPS etc.) have been developed. Although, RFLP and AFLP showed successful application for reporting genetic informations in plant species (Vos *et al*., 1995; Xu *et al*., 2000), but both these markers are still incapable of measuring the genetic distinctness in large populations (Talebi *et al*., 2008). Whereas, RAPD and SSR markers have overcome such difficulties and are highly polymorphic, suitable for proper DNA quantification (Williams *et al*., 1990).

2.6 Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is relatively easy technique which does not require nucleotide sequence information and is more polymorphic in detecting genetic diversity in plants (Ratnaparkhe *et al*., 1998). Polymorphisms usually caused by the difference occur in a nucleotide sequence (point mutations), or by rearrangement of the nucleotide sequence (Welsh and McClelland, 1990; Williams *et al*., 1990). In plant sciences RAPD analysis has been used in many applications among various organisms (Caetano-Anolles, 1994; Sharma and Mohapatra, 1996). The identification of many crops has been facilitated by RAPD technique (Mignouna *et al*., 1998). RAPD technique is highly polymorphic for studying genetic diversity in chickpea (Ratnaparkhe *et al*., 1998), its gene tagging (Rajesh *et al*., 2002), phylogenetic (Iruela *et al*., 2002),) and evolutionary studies (Reddy *et al*., 2002). Talebi *et al*. (2008) also investigated genetic diversity

among the Iranian elite chickpea genotypes by using RAPD markers and morphological traits. Similar studies were conducted by Ahmad *et al*. (2010) and Mahmood *et al*. (2011) by using Pakistani cultivars and proposed RAPD markers for selection of desirable chickpea germplasm. Agraval and Srivastava, (2010) assessed the genetic diversity in Indian chickpea cultivars using RAPD markers.

2.7 Microsatellites or simple sequence repeats (SSRs)

Microsatellites or simple sequence repeats are PCR based markers, they need nucleotide sequence information and have been reported by many researchers using a number of plants such as *Saccharum* (Cordeiro *et al*., 2001), *Oryza* (Cho *et al*., 2000),), *Hordeum* (Thiel *et al*., 2003), *Triticum* (Gupta *et al*., 2003), *Coffea* (Aggarwal *et al*,. 2007), *Citrus* (Chen *et al*., 2006) etc. The use of these markers was found to be useful by Varshney *et al*., 2005a in detecting cross-transferability and genetic diversity across closely related genera and species. Moreover, for QTL mapping of agronomically valuable traits such markers provide a source of direct gene tagging (Choudhary *et al*., 2008).

The currently available microsatellite or SSR (simple sequence repeat) markers are often chosen because of their co-dominant inheritance, multi-allelic nature and higher genome coverage (Gupta and Varshney, 2000). As a result a large number of SSR markers have been studied and developed for chickpea assessment (Winter *et al*., 1999; Huttel *et al*., 1999; Sethy *et al*., 2003; Lichtenzveig *et al*., 2005; Choudhary *et al*., 2006, Upadhaya *et al*., 2008). SSRs are considered highly polymorphic, thus easily transferable between populations and used to discriminate even closely related lines from each other (Gupta *et al*., 1999). SSRs are indeed excellent for studies of gene mapping and population genetics due to their co-dominant nature (Jarne and Lagoda, 1996; Goldstein and Schlotterer, 1999). In molecular breeding activities the molecular markers and genetic linkage maps are the prerequisites for yield improvement. In this way, the progress in development of molecular markers has been very slow in cultivated chickpea (*Cicer arietinum*). The main reason of which is a low level of genetic diversity in the gene pool of cultivated chickpea as reported by Varshney *et al*., 2007. The genome-

assisted breeding is still not that much successful to expose the potential of chickpea for cross-genome comparisons because of unavailability of knowledge and infrastructure (Varshney *et al*., 2009a). Nayak *et al*. (2010) also reported the development of single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers in chickpea. According to other observations DNA fingerprinting is one of a reliable tool for exploitation of potential usefulness of chickpea cultivars and can be used for delimiting the ideal lines (Jomova *et al*., 2009; Castro *et al*., 2010). Similar findings were also presented by Khan *et al*. (2010) as they used DNA fingerprinting techniques for screening the induced mutation lines and hybrid lines of chickpea germplasm to find the extent of genetic variations and relatedness between these lines.

In chickpea, seed size is one of the important growth parameter described by (Narayanan *et al*., 1981; Vadivelu and Ramakrishnan, 1983; Dahiya *et al*., 1985). Upadhyaya *et al*., 2006, also proposed the idea of direct relationship of seed size with seed weight. Therefore, genetical studies and understanding of the pattern of inheritance of traits in crop plants are required to develop seed of a specific size to meet market demand (Hossain, 2010). A great variation exists in seed size of chickpea desi and kabuli types, but sometimes kabuli types appear as small as the size found in desi type and the latter is attained larger size of kabuli type (Kumar and Singh 1995).

2.8 Screening of chickpea resistant lines against *Fusarium* wilt disease

Chickpea wilt disease is one of the most serious and major constrains which reduces the yield and production of crop in Pakistan (Ansar *et al*., 2010). *Fusarium* wilt of chickpea is seed-borne and seeds harvested from wilted plants when mixed with healthy seeds can carry the wilt fungus to new areas and can establish the disease in the soil to economic threshold levels within three seasons (Pande *et al.*, 2007). The disease occurs at seedling and lowering stage of plant growth. The symptoms which can be observed are drooping of petioles and rachis, yellowing and drying of leaves from base to upward, browning of vascular bundles, improper branching, withering of the plants and finally death of plants (Westerlund *et al.*, 1974; Prasad and Padwick, *et al.*, 1939). Pathogens enter the xylem vessels and invade the whole vascular system, inducing

symptoms of yellowing and wilting. In the absence of host plant the pathogen can survive up to six years (Haware, 1993).

Chauhan (1962) reported the initial symptoms of the disease due to pathogen infection to be vein clearing of leaves and decrease in the chloroplast and starch formation in mesophyll cells. Whereas, Erwin (1957) characterized chickpea wilt by yellowing of leaves and necrosis of the xylem. Leaves of the wilted plants turned greyish green, then became dull yellow and wilted. The xylem and pith become darkened and discolored. Moreover, internal discoloration of pith and xylem can be seen if the stem and root of the wilted plants split vertically (Saxena and Singh, 1987). The disease results in reduced plant population, reduced spear size and sub-optimal yield (Ravikumar *et al.*, 2007).

Mosahebi (1968) described that the fungus attacks the plants directly or indirectly through wounds made by nematodes and insect larvae. A general yellowing of the leaves and discoloration of vascular elements were the main symptoms of the disease. Later on Grewal (1969) reported two phases of wilt, the first phase being prominent at the seedling stage and the second at flowering and pod formation stage. Moreover, Nene *et al.* (1980) after making detailed symptomatolgical studies observed diagnostic symptoms of wilt at seedling stage (3-5 weeks after sowing) and the seedlings then collapsed and lay flat on the ground surface.

Murumkar and Chavan (1985) described physiological changes taking place in leaves infected by the fungal pathogen. The fungus attacks the root system made its way through the epidermis, cortex and finally penetrates into the xylem vessels of the tap root from where it spreads further. As a result, the lateral roots wither away. In many cases xylem vessels have been found to contain fungus mycelium, interfering with normal translocation of the sap. Seeds harvested from wilted plants were lighter in weight, rough (wrinkled surface) and dull in colour as compared with those obtained from healthy plants.

Datta and Lal (2011) used RAPD and SSR markers for the selection and

identification of resistant and susceptible chickpea lines. Gaur *et al*., (2006) recognized highly resistant genes for wilt disease in extra-large kabuli chickpeas. Whereas, Ravikumar and Ratna, (2007) found 15ppm concentration of fusaric acid more suitable for avoiding the disease symptoms. The linkage map of wilt resistant genes linked with sequence tagged markers is useful to check the least and higher efficiency of genes against wilt causing pathogens *i.e*., from *FOC* 0- *FOC* 5 races (Sharma and Muehlbauer, 2007). In previous studies the linkage map of resistance genes for *FOC* 1-5 races was developed using different RAPD and SSR markers in recombinant inbred lines (RILS) populations generated from various resistant and susceptible parental combinations (Winter *et al*., 2000; Sharma *et al*., 2004; Iruela *et al*., 2007; Gowda *et al*., 2009). Ansar *et al*. (2010) screened chickpea for resistance and susceptibility to wilt disease both in control and field conditions to distinguish resistant cultivars from susceptible ones. In chickpea wilt resistance is conferred by a single recessive allele which can be transferred through hybridization and pedigree selection into susceptible parents (Mahmood *et al*., 2011). Gayatri *et al*. (2012) also suggested such a defense mechanism against *Fusarium* wilt for chickpea lines to avoid the disease to some extent. Wilt disease cannot be feasibly controlled by fungicides because of its seed and soil born nature. Therefore, resistant cultivars should be used which is the cheapest way for chickpea wilt disease inhibition and management (Nene and Haware *et al*., 1980; Nene and Reddy 1987; Bakhsh *et al*., 2007; Ansar *et al*., 2010). Furthermore, Pranjan *et al*. (2010) constructed a phylogenetic tree to define the chickpea functional genes sequence relationship with other genes.

MATERIALS AND METHODS

3.1 Plant material

Seventy accessions of chickpea germplasm were obtained from Gene bank of Plant genetic resource institute (PGRI), National agriculture research centre, Islamabad, Pakistan; out of which twenty four accessions were indigenous and forty six acquired from (USA) (Table 3.1). Twenty four indigenous accessions of chickpea representing all the chickpea growing areas of Pakistan were included in the investigation. The Figure (3.1) represents the distribution of chickpea germplasm evaluated in the present study. More than 90% of chickpea is cultivated in Punjab followed by the Sindh province, hence the germplasm collected from these areas were investigated. These accessions represented the germplasm conserved in the genebank because these accessions were selected from different clusters in a previous study conducted by Gulbaz (2002). Other germplasm from USDA was included in the study for the comparison between exotic and indigenous genetic resources of chickpea, however the germplasm from USDA was evaluated for the first time in the present study.The accessions were sown in the agricultural field of department of Botany, University of Malakand, Khyber Pakhtunkhwa, Pakistan in randomize complete block design (RCBD). The experimental sites were located in the vicinity of mountains spanning between 34° 40' North, latitude and 72° 03' East longitudes between an elevations ranged from 728 to 735 m above mean sea level (amsl). The accessions were planted and set in duplicated manner followed the standard techniques of randomized complete block design (RCBD), keeping a row distance of 75cm with row length of 5m respectively (Clewer and Scarisbrick (2001). The culturing practices recommended by other workers were followed accordingly in cropping season in order to achieve avigorous and healthy crop. The accessions were allowed for testing under field conditions during three consecutive years, *i.e.*, 2009, 2010 and 2011 respectively (Sowing during November and harvesting in April for all the cropping season).

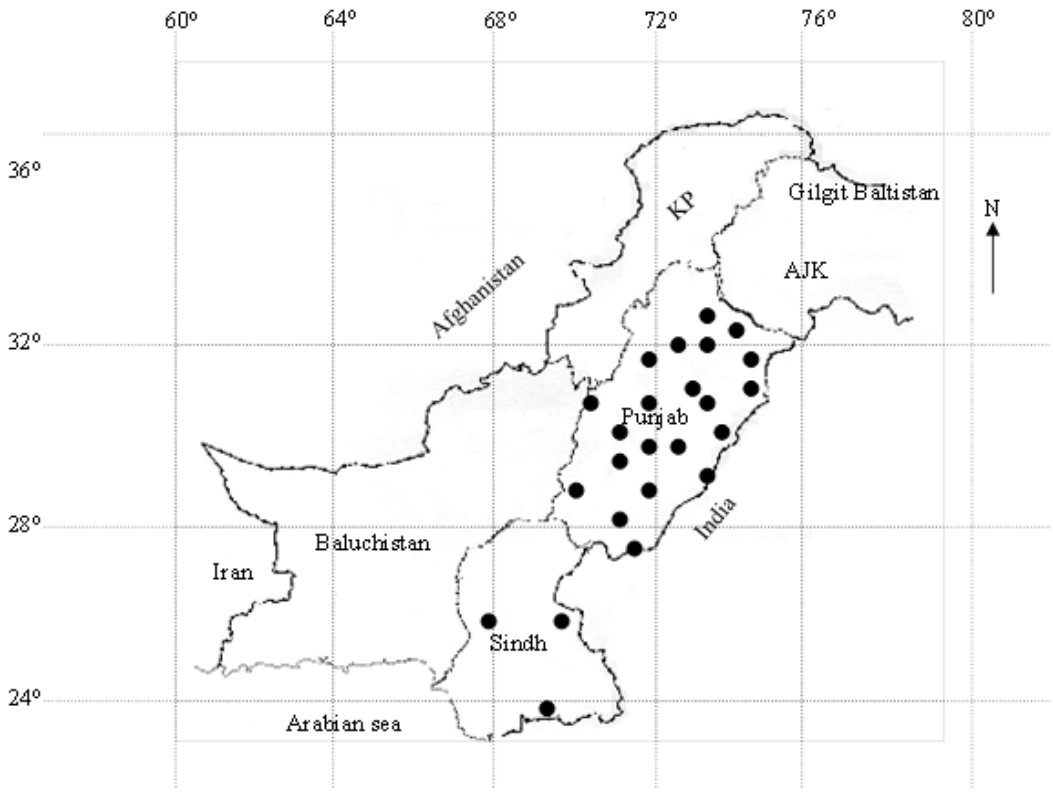


Fig. 31a :- Indigenous chickpea germplasm collection sites (●) in Pakistan.

3.2 Morphological characterization

The obtained accessions of chickpea were characterized on the basis of qualitative and quantitative traits (Table 3.1 and 3.2) in morphometric studies. Recording morphological characters followed the procedure described by chickpea descriptor (IBPGR, 1985; IBPGR, ICRISAT & ICARDA, 1993) with minor modifications.

The following qualitative and quantitative morphological traits analysis, ten competitive plants were sampled to record the data.

3.2.1 Qualitative morphological traits

1. Growth habit: Observed growth habit of the plant and rated as 1= Erect; 2= Semi- erect and 3= Prostrate

2. Plant vigor: Observe Plant vigor of the plant and rated as 1= Vigorous; 2= Very vigorous; 3= Weak; 4= Very weak

3. Flower color: Observed color of flowers of the plant and rated as 1= Purple; 2= Creamy; 3= Bluish purple; 4= Reddish purple; 5= Light purple

4. Seed color: Observed color of seeds of the plant and rated as 1= Yellow; 2= Green brown; 3= Brown; 4= Light brown; 5= Reddish brown; 6= Dark brown; 7= Grey; 8= Black (Table 3.2).

3.2.2 Quantitative morphological traits

1. 100 Seed weight: Selected randomly 100 seeds from the bulk of seeds and weighed in gram. The mean value was then computed.

2. Grain yield: Ten plants were randomly selected from each accession after maturity. They were weighed. Mean was then computed.

3. Total biomass: Dry weight (gm) of ten randomly selected plants was recorded.

4. Harvest index (%): Ratio of grain yield and biomass per plant multiplied by 100. HI (%) = (Grain yield/ biomass) × 100

Table 3.1: Chickpea 70 indigenous and exotic accessions

S/ No.	Accession No.	Country of origin	Province	City	S/No.	Accession No.	Country of origin
1	1898	Pakistan	Sindh	Jacobabad	36	3015	USA
2	1936	Pakistan	Punjab	Muzaffargarh	37	3016	USA
3	1995	Pakistan	Punjab	Faisalabad	38	3017	USA
4	1998	Pakistan	Sindh	Thatta	39	3020	USA
5	2023	Pakistan	Sindh	Mirpur Khas	40	3021	USA
6	2188	Pakistan	Punjab	Bhakkar	41	3022	USA
7	2234	Pakistan	Punjab	Faisalabad	42	3023	USA
8	2235	Pakistan	Punjab	Faisalabad	43	3024	USA
9	2236	Pakistan	Punjab	Faisalabad	44	3026	USA
10	2237	Pakistan	Punjab	Faisalabad	45	3027	USA
11	2272	Pakistan	Punjab	Faisalabad	46	3031	USA
12	2273	Pakistan	Punjab	Faisalabad	47	3032	USA
13	2278	Pakistan	Punjab	Faisalabad	48	3033	USA
14	2430	Pakistan	Punjab	Jhang	49	3035	USA
15	2441	Pakistan	Punjab	Bhakkar	50	3037	USA
16	2473	Pakistan	Punjab	Chakwal	51	3039	USA
17	2497	Pakistan	Punjab	Attock	52	3040	USA
18	2499	Pakistan	Punjab	Attock	53	3041	USA
19	2531	Pakistan	Punjab	Khushab	54	3042	USA
20	2532	Pakistan	Punjab	Mianwali	55	3043	USA
21	2544	Pakistan	Punjab	Khushab	56	3044	USA
22	2553	Pakistan	Punjab	Bhakkar	57	3045	USA
23	2558	Pakistan	Punjab	Layyah	58	3046	USA
24	2562	Pakistan	Punjab	Layyah	59	3047	USA
25	2595	USA	---------	---------	60	3054	USA
26	2611	USA	---------	---------	61	3056	USA
27	2616	USA	---------	---------	62	3057	USA
28	2629	USA	---------	---------	63	3058	USA
29	2650	USA	--------	---------	64	3059	USA
30	2654	USA	--------	---------	65	3061	USA
31	2819	USA	--------	---------	66	3062	USA
32	2831	USA	--------	---------	67	3063	USA
33	2855	USA	--------	---------	68	3064	USA
34	2859	USA	--------	--------	69	3065	USA
35	3011	USA	--------	--------	70	3066	USA

Table 3.2: Codes used for various qualitative morphological traits in present study

S/ No.	Traits	Statistical Codes
1	**Growth habit**	
	i. Erect	1
	ii. Semi erect	2
	iii. Prostrate	3
2	**Plant vigor**	
	i. Vigorous	1
	ii. V.vigorous	2
	iii. Weak	3
	iv. V.weak	4
3	**Flower color**	
	i. Purple	1
	ii. Creamy	2
	iii. Bluish purple	3
	iv. Reddish purple	4
	v. Light purple	5
4	**Seed color**	
	i. Yellowish white	1
	ii. Green brown	2
	iii. Brown	3
	iv. Light brown	4
	v. Reddish brown	5
	vi. Dark brown	6
	vii. Grey	7
	viii.Black	8

To check the inference of seed size (length and width) upon seed weight, the samples of seeds were categorized into three classes: (i) Small (ii) Medium (iii) and large size based on the length and width of each seed and the size was measured by the procedure defined by Ahirwar, 2012. According to which the data obtained in centimeter (cm) was converted into millimeter (mm) for valid comparison. The samples of seeds were categorized into different size ranges from 3mm to 9.9mm.

3.2.3 Multiplication of inoculum

Mass culture of the fungus was prepared by soaking sorghum grains in tap water overnight and than surface dried by spreading on paper towels in laboratory under a ceiling fan. Surface dried seeds were put into conical flasks @ 250 g flask-1 and the flasks were closed by inserting cotton plugs. These flasks were autoclaved at 15 psi for 20 minutes. The sterilized flasks after cooling were inoculated with ten days old *F. oxysporum* f. sp. *ciceris* actively growing culture, by adding 4 mm agar plugs using a sterile cork borer. After plugging these flasks were incubated at 26±2o C for 10 days. At the time of inoculation, each of the test isolates was mixed thoroughly to develop wilt sick beds where the accessions were plotted in rows for further experiment.

3.2.4 Screening of chickpea for wilt resistance

To observe the resistant chickpea accessions against *Fusarium* wilt, the screening of chickpea germplasm of indigenous and exotic origin was made under field and greenhouse conditions during the years 2012 and 2013 respectively. These genotypes were screened in wilt sick beds at the department of Botany, University of Malakand, Khyber Pakhtunkhwa, Pakistan.

3.2.5 Disease evaluation

Chickpea germplasm was tested for wilt resistance against *F. oxysporum* f. sp. *ciceris* (*FOC*) using the isolates provided by the department of Pathology, University of the Punjab. The fungal inoculum was increased by multiplying with sorghum grains. There are different disease rating scales (Tullu, 1996; El-Hadi, 1993; Jimenez-Diaz *et al*., 1989; Phillips, 1988; Haware and Nene, 1982,) but these lines were screened according to the rating scales given by Iqbal *et al*., (2005).

3.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an important economic and consistent tool for the estimation of genetic diversity and crop improvement (Ghafoor *et al*., 2002; Javid *et al*., 2004; Nisar *et al*., 2009). To achieve this goal SDS-PAGE was used for the determination of genetic diversity on the basis of

biochemical analysis. The electrophoresis was carried out in a Slab type SDS-PAGE using Japanese apparatus (Model: AE-6530M), with 12% (w/v) polyacrylamide gel, in a discontinuous buffer system following the method outlined by Laemmle (1970). For the extraction of proteins, a single seed was ground to fine powder with mortar and pestle. Protein extraction buffer (PEB) 400µl was added to 0.01g of seed flour and vortexed thoroughly to homogenize. In order to purify, the homogenate samples were centrifuged at 12,000 rpm for 10 minutes at room temperature. The isolated crude proteins were recovered as clear supernatant, transferred into new 1.5 ml E-tube and stored at 2ºC until further analysis. About, 15 µl of the sample supernatant was loaded for protein separation. The apparatus was connected to a constant electric supply (100 V) until the bromophenol blue reached the bottom of the gel plates.

3.4 Molecular characterization

A. DNA extraction and purification

Dry seeds were selected for the isolation of genomic DNA through a modified technique of Kang *et al*., (1998). The DNA was quantified with the help of spectrophotometer (Manning, 1991). The following steps were taken for the extraction of DNA:

1. Single seed was ground in a microcentrifuge tube (1.5 ml)
2. 400 µl extraction buffer (200mM Tris-HCL (pH 8.0), 25mM EDTA, 200mM NaCl, 0.5% (w/v) SDS containing 50 µl of Proteinase K) was added in microcentrifuge tube.
3. The tubes were incubated at 37 ^{o}C for one hour and then homogenized with a glass rod.
4. 400 µl of 2% (v/v) CTAB solution (100mM Tris-HCl (pH 8.0), 20mM EDTA (pH 8.0), 1.4M NaCl, 2% CTAB (w/v), 1% PVP (polyvinylpyrrolidone 40,000) was added to the incubated sample.
5. In addition 400 µl chloroform: isoamyl alcohol (24:1) with 5% (w/v) phenol was added.
6. The tubes were centrifuged at 12000 rpm at 4 ^{o}C for 10 minutes and

transffered the supernatant to new tubes (pellet was discarded).

7. Added 2/3 volume Isopropanol and the tubes were then incubated at room temperature for 10 minutes to precipitate DNA.
8. The tubes were centrifuged at 12000 rpm for 5 minutes.
9. Supernatant was removed and the pellet was washed with 70% (v/v) ethanol and then centrifuge at 12000 rpm for 5 minutes at room temperature and 70% (v/v) ethanol discarded from the tube.
10. Air dry DNA pellet in vacuum drier for 5-10 minutes and then the pellet re-suspended in 50 µl TE buffer.
11. Finally 2 µl of Rnase (10mg/ml) was added to remove the RNA from dissolved DNA

B. DNA quantification

The DNA was quantified with the help of spectrophotometer at 260 nm optical density. The genomic DNA was run on a 1% (w/v) agarose gel dissolved in 1xTBE stained with ethidium bromide and the gels visualized on a trans-UV and photographed with Bio-Rad gel documentation system for PCR reaction.

3.5 PCR reaction

To optimize the conditions for Polymerase chain reaction (PCR) 25µl of reaction mixture was prepared. For PCR reproducibility 2X concentrated solution of PCR master mixture (0.05µl *Taq* DNA polymerase, Reaction buffer, 4mM $MgCl_2$ and 0.4mM of each dNTP) was used in the reaction. The different combinations of which were checked for maximum amplification as shown in table (3.3). Thermal cycling was optimized with denaturation temperature for two minutes at 94^0C, annealing temperature for 1 minute at 37^0C (RAPD) and 55^0C (SSR), while, extension temperature 72^0C for 7- 10 minutes using RAPD and SSR primers respectively (Table 3.4). The PCR product was resolved on a 2% (w/v) agarose gel in 1x TBE buffer at 100 V. Tracking dye was mixed in PCR tube (containing mastermix) and mixed well. The PCR product was run and visualized the DNA profile under gel documentation system for the scoring of data for linkage analysis.

Table 3.3: Formulation for the PCR reaction (RAPD and SSR)

S.No.	Chemicals	Required quantity
1.	PCR master mixture	12.5µl
2.	Primer (RAPD) Primer forward/ primer reverse (SSR)	2 µl 1 µl/ 1µl
3.	ddH2O	8.5 µl
4.	Genomic DNA	2 µl

Table 3.4: Thermo cyclic conditions for RAPD and SSR optimized for chickpea germplasm

S/ No.	Conditions optimized for SSR	Conditions optimized for RAPD	Cycle Timing in minutes
1	94^0C	94^0C	2
2	94^0C	94^0C	1
3	55^0C	37^0C	1
4	72^0C	72^0C	2
5	Repeated 2-4, 40 cycles	Repeated 2-4,40 cycles	
6	72^0C	72^0C	7/ 10

3.6 Agarose gel electrophoresis

After electrophoresis the gels were stained with 0.2% (W/V) Coomassie Brilliant Blue dissolved in 10% (V/V) acetic acid, 40% (V/V) methanol and water in the ratio of 10:40:50 (V/V) for about one hour. Gels were destained in a solution containing 5% (V/V) acetic acid and 20% (V/V) methanol. Gels were shake gently to make the background of the gel protein visible. The destained gels were then read either by direct photographic method (FA 500 EPI-Light UV gel documentation system) or by drying the gels on sheets using gel-drying processor for about 2-4 hours.

3.7 Genetic diversity

The accessions were tested through 20 RAPD (Table 3.5) and 20 SSR makers (Table 3.6) for estimation of genetic diversity in the collected lines based on loci presence and absence.

Table 3.5: Sequences of the RAPD primers used in the present study for molecular analysis of chickpea germplasm

S/No.	Primer	Sequence (5'-3')	S/No.	Primer	Sequence (5'-3')
1.	UBC 181	ATGACGACGG	11.	OPA10	GTGATCGCAG
2.	UBC 733b	GGGAAGGGAG	12.	OPB11	GTAGACCCGT
3.	OPA4	AATCGGGCTG	13.	OPB12	CCTTGACGCA
4.	OPA9	GGGTAACGCC	14.	OPB13	TTCCCCCGCT
5.	OPG13	CTCTCCGCCA	15.	OPB14	TCCGCTCTGG
6.	OPA1	CAGGCCCTTC	16.	OPB15	GGAGGGTGTT
7.	OPA2	TGCCGAGCTG	17.	OPB16	TTTGCCCGGA
8.	OPA3	AGTCAGCCAC	18.	OPB17	AGGGAACGAG
9.	OPA6	GGTCCCTGAC	19.	OPB18	CCACAGCAGT
10.	OPA7	GAAACGGGTG	20.	OPB19	ACCCCCGAAG

Table 3.6: Sequences of the SSR primers used in the present study for molecular analysis of chickpea germplasm

S/ No.	Primer Name	Sequence forward/ Reverse	No. of bands	Molecular weight bp
1	CaSTMS2	ATTTTACTTTACTACTTTTTTCCTTTC AATAAATGGAGTGTAAATTTCATGTA	2	114
2	CaSTMS15	CTTGTGAATTCATATTTACTTATAGAT ATCCGTAATTTAAGGTAGGTTAAAATA	1	159
3	CaSTMS21	CTACAGTCTTTTGTTCTTCTAGCTT ATATTTTTTAAGAGGCTTTTGGTAG	1	60
4	TA72	GAAAGATTTAAAAGATTTTCCACGTTA TTAGAAGCATATTGTTGGGATAAGAGT	1	198
5	TA130	TCTTTCTTTGCTTCCAATGT GTAAATCCCACGAGAAATCAA	1	219
6	TA194	TTTTTGGCTTATTAGACTGACTT TTGCCATAAAATACAAAATCC	2-3	204
7	TA71	CGATTTAACACAAAACACAAA CCTATCCATTGTCATCTCGT	1	202
8	TA22	TCTCCAACCCTTTAGATTGA TCGTGTTTACTGAATGTGGA	1	228

9	TA200	TTTCTCCTCTACTATTATGATCACCAG TTGAGAGGGTTAGAACTCATTATGTTT	1	296
10	TA46	TTTATTGCAATAAAACTCATTTCTTATC TTCTTTTTGTGTGAAAAAAAAATATAGTA	1	239
11	TA135	TGGTTGGAAATTGATGTTTT GTGGTGTGAGCATAATTCAA	1	192
12	TR1	CGTATGATTTTGCCGTCTAT ACCTCAAGTTCTCCGAAGT	1	224
13	TR7	GCATTATTCACCATTTGGAT TGTGATAATTTTCTAAGTGTTTT	1	204
14	TR29	GCCCACTGAAAAATAAAAAG ATTTGAACCTCAAGTTCTCG	2	220
15	TR31	CTTAATCGCACATTTACTCTAAAATCA ATCCATTAAAACACGGTTACCTATAA	1	217
16	RM104	GGAAGAGGAGAGAAAGATGTGTGTCG TCAACAGACACACCGCCACCGC	----	222
17	RM244	CCGACTGTTCGTCCTTATCA CTGCTCTCGGGTGAACGT	----	163
18	RM6836	TGTTGCATATGGTGCTATTTGA GATACGGCTTCTAGGCCAAA	----	240
19	RM8225	TGTTGCATATGGTGCTATTTGA GATACGGCTTCTAGGCCAAA	----	221
20	RM206	CCCATGCGTTTAACTATTCT CGTTCCATCGATCCGTATGG	----	147

3.8 Marker trait association/ correlation

A. Marker assisted selection (MAS) for seed size and seed weight

The molecular markers were linked to the genes responsible for 100 seed weight and seed size (Hossain, 2010; Joshi *et al*., 2010; Castro *et al*., 2010) using Kang *et al.*, 1998 described protocol.

B. Marker assisted selection (MAS) for *Fusarium oxysporum* wilt resistance

In order to further evaluate the field observations for *Fusarium* wilt resistant lines the RAPD and SSR markers were correlated to *Fusarium* wilt resistance gene that was

previously described in detail by various researchers (Sharma *et al*., 2003; Iruela *et al*., 2007; Sharma and Muehlbauer, 2007; Datta *et al*., 2010; Soregaon and Ravikumar, 2010). The DNA was quantified with the help of spectrophotometer (Manning, 1991).

3.9 Data analysis

3.9.1 Morphological data analysis

i. Genetic diversity

The data scored for the determination of genetic diversity of morphological traits were subjected to statistical analysis including simple statistics (Mean, standard error, standard deviation, coefficient of variation (% age) with minimum and maximum ranges), correlation coefficient and frequency distribution of three years data for environmental error.

ii. Yield related traits

The data obtained for 100 seed weight and seed size were subjected to statistical analysis to find the level of genetic correlation of seed size/ seed weight, frequency distribution (%) and cumulative frequency of seed size categories based on length and width of each accession.

iii. Disease screening

The observations were made in rates (%) of accessions showed wilting at seedling stage, flowering time and complete response till pods maturity by using the wilt incidence formula (Mehrotra and Aggarwal, 2003).

$$\text{Wilt incidence (\%)} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}} \times 100$$

The degree of susceptibility and resistance to disease of each line was determined by using, 1-9 rating scale given by Iqbal *et al.*, (2005), where,

1 Highly resistant = Less than 1% of plant wilted.

3 Resistant = 1-10% of plants wilted.

4 Moderately resistant = 11-20% of plants wilted.

7 Susceptible = 21-50% of plants wilted.

9 Highly susceptible = 51% or more of plants wilted.

3.9.2 SDS-PAGE data analysis

In case of SDS-PAGE and RAPD, SSR markers analysis the presence (1) and absence (0) of bands was entered in a binary data matrix. Based on appearance the visible bands were considered as major and lighter as minor. To check the genetic diversity and rough estimation of the location of each band starting from 1-16 run a molecular weight marker once with few accessions and then applied for all gels. The experiment was performed twice to re-identify the location and reproducibility. The presence (1) and absence (0) of bands were then used to construct the dendrograms for the estimation of genetic disagreement to calculate the genetic diversity among local and exotic accessions.

3.9.3 Molecular makers data analysis

A comparison of the seventy local and exotic accessions of *C. arietinum* L. was performed on the basis of the presence or absence of bands generated by the SSR and RAPD primers. The number of bands produced for each primer was scored manually for presence (1) or absence (0). For estimation of linkage distance the scored data were put in a binary data matrix to develop a dendrogram based on un-weighted pairs group mean average (UPGMA) already used by Dahab *et al*., 2013. The genetic distances among the accessions were calculated by the percentage disagreement method to assess the similarity and genetic relationship of chickpea accessions using the statistical software package STATISTCA- ver.6 (StatSoft, Inc., 2001).

3.9.4 Marker trait association analysis

Data of both RAPD and SSR makers were scored and the binary data matrixes were developed. The matrix was subjected for statistical analysis *i.e*., Standard deviation, *t*-test and correlation coefficient. For correltion analysis the matrix was analyzed using cluster analysis and Two-way Joining Tree (McCune & Grace, 2002).

3.9.5 Marker assisted selection (MAS) for *Fusarium oxysporum* wilt resistance

The data from electrophorogram were scored by the presence (1) an absence (0) of allele. The variation intensity was not taken in consideration, but the association of molecular marker with wilt was scored. On the basis of presence and absence of alleles, cluster analysis of 70 lines was performed to sort the lines with response to disease status.

Coefficient of similarity based on UPGMA was performed. For Pearson correlation *t*-test (alpha ≤ 0.05) was applied using STATISTICA version 7 for windows. The probability of molecular markers was estimated and confirmed through receiver operating characteristic curve (ROC) analysis. All of the other analyses however were made by statistical software "STATISTICA version 6" (Nisar *et al*., 2008).

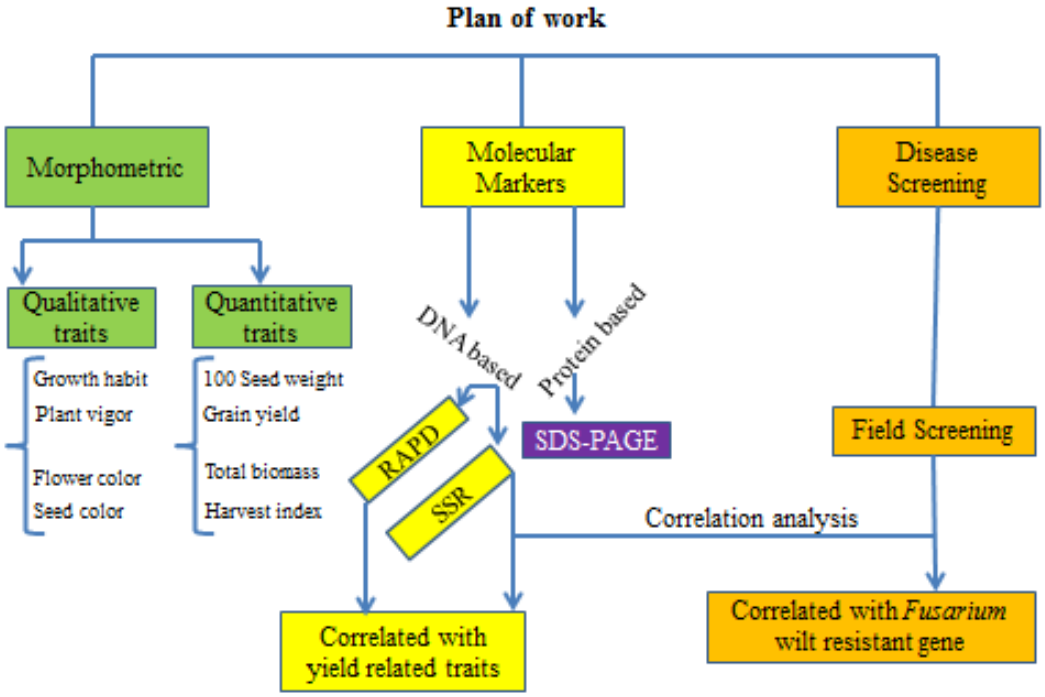


Figure 3.1: Graphical representation of work plan for the present project

RESULTS

The experimental results of morphological, biochemical and molecular markers data analyses have been presented as follows:

4.1 Morphological characterization

To document the level of morphometric variation in accessions, the plants were assessed for morphological characters. These were classified as qualitative and quantitative traits:

4.1.0 Qualitative traits

Four qualitative traits *i.e.*, growth habit, plant vigor, flower color and seed color were studied for frequency distribution (Table 4.1, Figure 4.7). The results showed that the average coefficient of variation for all groups of qualitative traits was scored as 44.8% (Table 4.2).

A. Growth habit

Three growth forms were observed in *Cicer* described as erect, semi erect and prostrate with frequency distribution for each class being 78.57%, 12.85% and 8.57% respectively (Figure 4.1). The mean value calculated for growth habit was 33.33 ± 22.65 with coefficient of variation 11.77% (Table 4.2).

B. Plant vigor

In the present study four states of plant vigor were observed: vigorous, very vigorous, weak and very weak with frequency distribution 57.1%, 34.28%, 7.14% and 1.42% in total chickpea germplasm respectively (Figure 4.2). The mean value of plant vigor was estimated as 24.99 ± 12.88 with 10.31% coefficient of variation (Table 4.2).

C. Flower color

The flower color in chickpea was as scored as: purple (42.85%), creamy (28.57%), bluish purple (7.14%), reddish purple (7.14%) and light purple (14.28%) (Figure 4.3). Notably, the coefficient of variation was scored as 77.41% for flower color with mean value 20.00 ± 6.92 (Table 4.2).

D. Seed color

In case of seed coloration a total of eight colors of seeds were recognized in chickpea germplasm in the present study i.e., yellowish white (34.28%), greenish brown (2.85%), pale yellow (7.14%), Brown (14.28%), light brown (15.71%), Reddish brown (4.28%), Dark brown (8.57%) and Black (12.85 %) (Figure 4.4).

The frequency (%) of all groups of selected qualitative traits of chickpea through a scattered plot with moving average line has been presented in Figure 4.5. A comparatively higher coefficient of variation (79.52) was calculated for seed color with mean value 12.50 ± 3.51 (Table 4.2).

Table 4.1: Frequency distribution of qualitative traits in chickpea

Trait	Groups	Codes	Plants (n=70)	Frequency (%)
1. Growth habit	Erect	1	55	78.57
	Semi erect	2	09	12.85
	Prostrate	3	06	8.57
2. Plant vigor	Vigorous	1	40	57.1
	V.vigorous	2	24	34.28
	Weak	3	05	7.14
	V.weak	4	01	1.42
3. Flower color	Purple	1	30	42.85
	Creamy	2	20	28.57
	Bluish purple	3	05	7.14
	Reddish purple	4	05	7.14
	Light purple	5	10	14.28
4. Seed color	Yellowish white	1	24	34.28
	Greenish brown	2	02	2.85
	Pale yellow	3	05	7.14
	Brown	4	10	14.28
	Light brown	5	11	15.71
	Reddish brown	6	03	4.28
	Dark brown	7	06	8.57
	Black	8	09	12.85

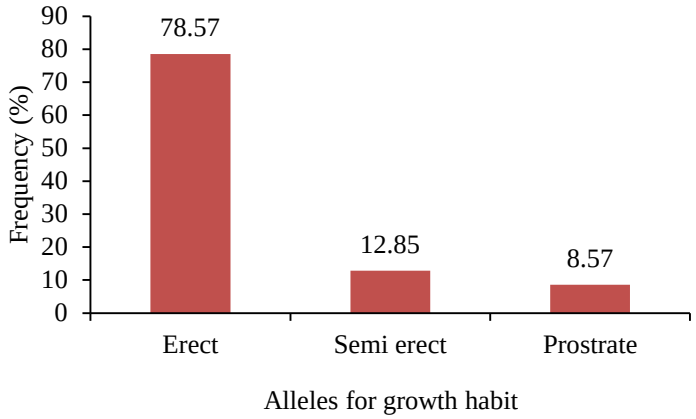


Figure 4.1: Frequency distribution for growth habit in chickpea 70 accessions

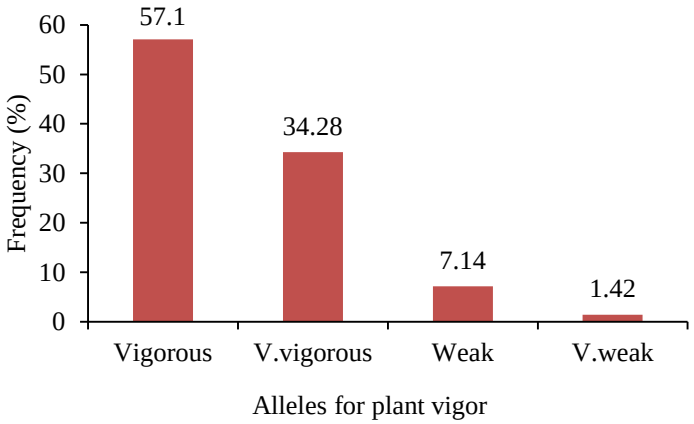


Figure 4.2: Frequency distribution for plant vigor in chickpea 70 accessions

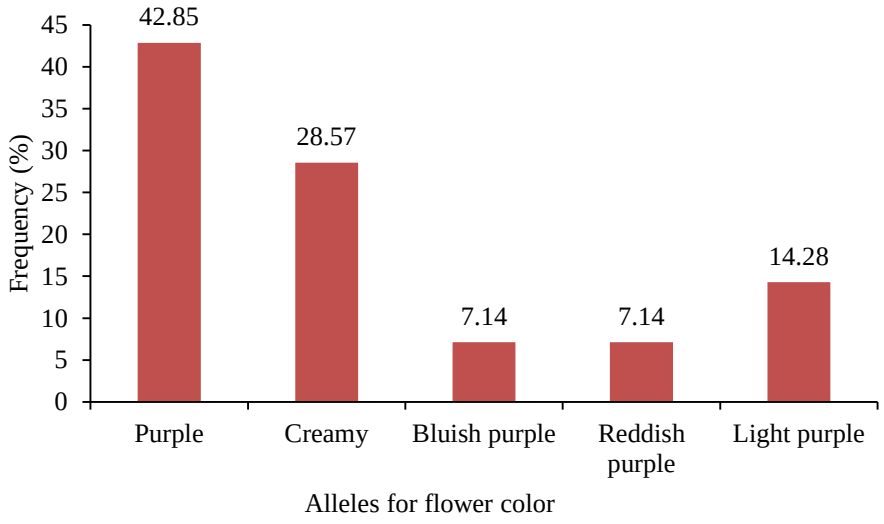


Figure 4.3: Frequency distribution for flower color in chickpea 70 accessions

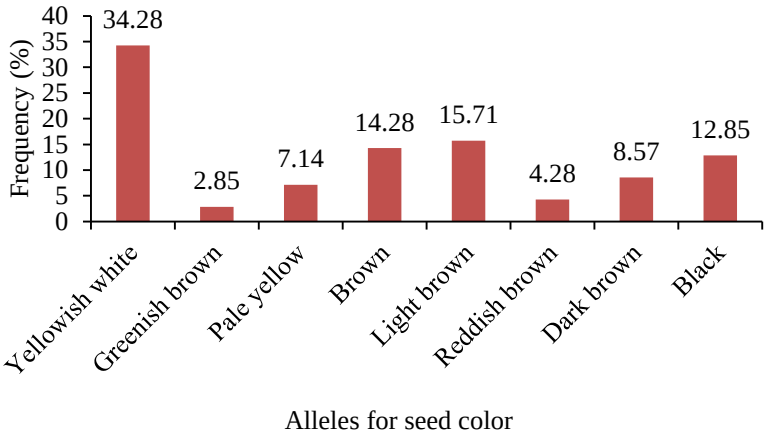


Figure 4.4: Frequency distribution for seed color in chickpea 70 accessions

Table 4.2: Basic statistics of qualitative traits studied in chickpea germplasm

Traits	Mean	S.E	Std.dev.	Minimum	Maximum	C.V%
Growth habit	33.33	22.65	39.24	8.57	78.57	11.77
Plant vigor	24.99	12.88	25.77	1.42	57.10	10.31
Flower color	20.00	6.92	15.48	7.14	42.85	77.41
Seed color	12.67	3.67	10.40	2.85	35.71	79.52

C.V%- Represent coefficient variation percentage
S.E - Standard error, Std.dev. - Standard deviation σ^2 Ava = 44.8%

4.1.1 Quantitative traits

The morphological data analysis based on four quantitative traits *viz.*, 100 seed weight, grain yield, total biomass and harvest index, revealed variation among chickpea accessions (Appendix 17-19).

A. 100 seed weight

The data analyzed for 100 seed weight showed a slight decline from 27.41 ± 1.32 and 27.70 ± 1.38 in 2008-2009 and 2010-2011 respectively to 26.94 ± 1.37 in 2009-2010 (Table 4.3).

B. Grain yield

The average variation of all accessions observed for grain yield was 90.20 ± 6.81 in 2008-2009, this value dropped to 83.82 ± 6.56 in 2009-2010 and 72.78 ± 7.92 in 2010-2011 (Table 4.3).

C. Total biomass

The total biomass improved from 442.71 ± 28.30 in 2008-2009 to 447.83 ± 32.16 g/5plants in 2009-2010 but again dropped to 424.8± 32.30 in 2010-2011 (Table 4.3).

D. Harvest index

Similarly, the mean value for harvest index dropped from 21.60 ± 1.34 in 2008-2009 to 20.75 ± 1.28 in 2009-2010 and 18.72± 1.62 in 2010-2011 (Table 4.3).

Table 4.3: Basic statistics of quantitative traits studied in chickpea germplasm

Traits	Mean	S.E	Std.dev.	Minimum	Maximum	C.V%
100/ Seed Weight	27.41 a 26.94 b 27.70 c	1.32 a 1.37 b 1.38 c	11.02 a 11.6 b 11.6 c	12.36 a 8.53 b 8.50 c	57.76 a 64.51 b 56.80 c	40.21 a 42.53 b 41.73 c
Grain Yield	90.20 a 83.82 b 72.78 c	6.81 a 6.56 b 7.92 c	56.97 a 54.89 b 66.23 c	1.20 a 7.10 b 10.50 c	287.6 a 316.7 b 228.9 c	63.15 a 65.49 b 91.00 c
Total Biomass	442.7 a 447.8 b 424.8 c	28.30 a 32.16 b 32.30 c	236.8 a 269.05 b 270.2 c	50.00 a 101.00 b 87.00 c	1150.00 a 1322.00 b 1422.00 c	53.48 a 60.08 b 63.61 c
Harvest Index	21.60 a 20.75 b 18.72 c	1.34 a 1.28 b 1.62 c	11.23 a 10.72 b 13.58 c	0.22 a 4.91 b 1.73 c	53.38 a 48.83 b 53.02 c	52.01 a 51.65 b 72.56 c

C.V%- Represent coefficient of variation percentage

a- Represent year 2008-2009 σ^2 Ava = 56.8%

b- Represent year 2009-2010

c- Represent year 2010-2011

4.2 Frequency distribution

When compared data for genotypes frequency distribution, a notable variation was observed for harvest index and seed weight, where 47.14% of the genotypes were placed in the frequency class ≤ 22.5-32.4 of 'seed weight' and ≤ 12.5-23.4 for 'harvest index' in 2010 (Figure 4.5). The overall estimated variation could be attributed to 5% environmental error (EE) for '100 seed weight revealing this to be a rather stable trait while 19% EE was recorded in 'harvest index'. The data obtained in 2009 showed more than 50% genotypes as one frequency class while this threshold could not be achieved for 2010-2011. Hence the pattern of genotype distribution varied much with a higher level (19%) variation attributed to environmental error. However, a different pattern was observed for grain yield where over half the numbers of genotypes *i.e.,* 51% of the total genotypes were placed in one frequency class (1.1-58.3) and the remaining of the accessions populated other frequency classes. Similarly more than 50% genotypes were also observed in a class, ranged from 1.1-58.3 in 2011, associated with 12% environmental error. While in case of total biomass in 2011 a comparatively high percentage of accessions *i.e.,* 48% and 36% were placed in frequency classes (50-279) and (280-509) respectively. On the other hand 44% of genotypes were placed in the same

class range in 2009 with 8% environmental error (Figure 4.6).

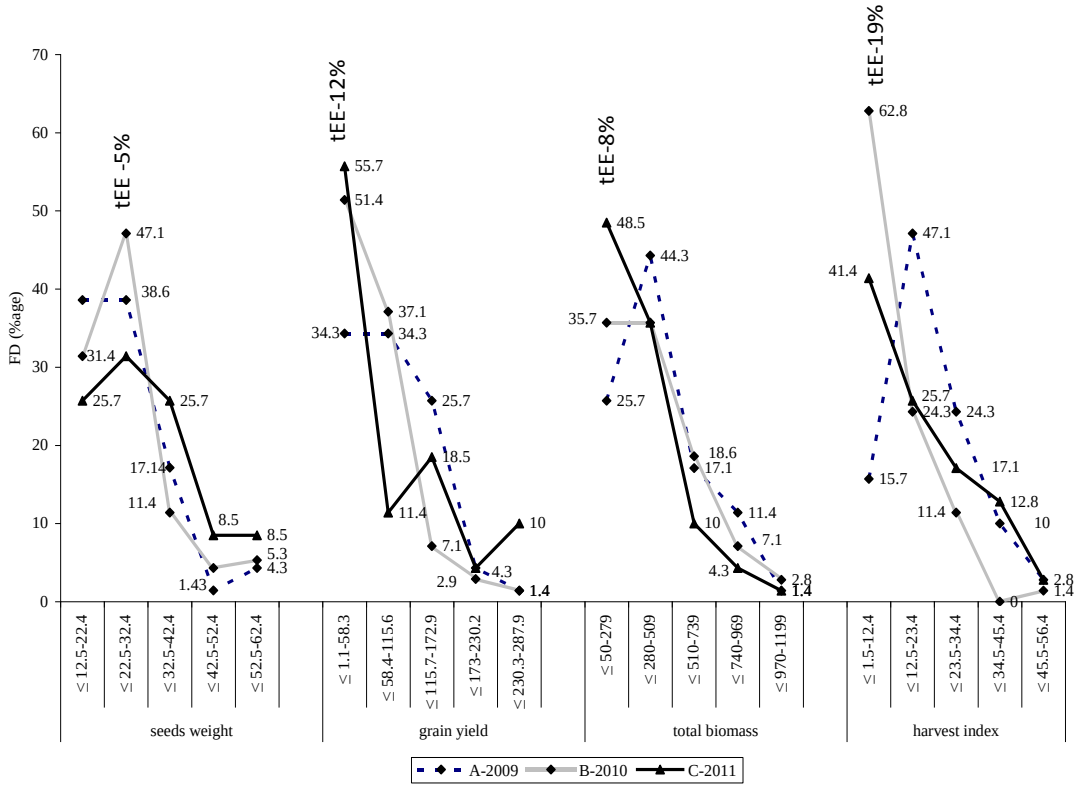


Figure 4.5: Comparative Picture showing frequency distribution of three years data collected during 2008 to 2011 of chickpea germplasm. tEE-total environmental error across the years

4.3 Selection of best accessions based on performance

Data analysis further showed that the accessions 3063, 3040, 3022, 3059 performed better for a single trait *i.e.,* 'total biomass'. The 'grain yield' performance was found best in the genotypes 2819 and 3039. However, accession 3056 was best for '100 seed weight' and 1898 for 'harvest index'. For two traits, the genotype 3037 performed better than other genotypes for 100 seed weight and total biomass. Similarly 3054 and 3043 were found best for three quantitative traits 'grain yield', 'total biomass' and '100 seed weight' (Table 4.4, figure 4.6).

Table 4.4: Selection of genotypes on the basis of their performance in chickpea genotypes

Traits	G-1	G-2	G-3	G-4	G-5	G-6	G-7
100 seed weight	3037**	3043***	3054***	3056*			
Grain yield	2819*	3039*	3043***	3054***			
Total Biom	3022*	3037**	3040*	3043***	3054***	3059*	3063*
Harvest Index	1898*	-----	-----	-----	-----	-----	----

G- Genotype; *- best performance for a single trait; **- best performance for two traits; ***- best performance for three traits

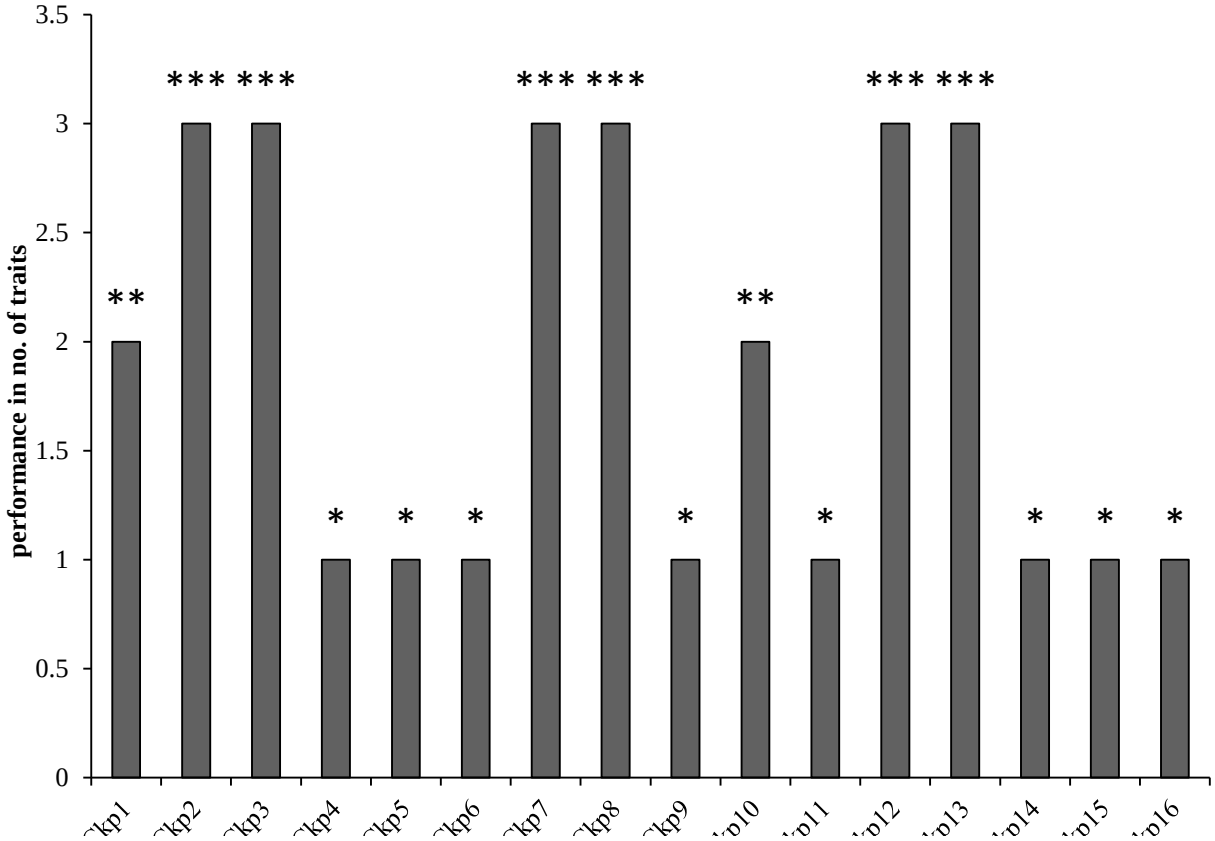


Figure 4.6: Three years performance comparison of chickpea selected genotypes. *- best performance for a single trait; **- best performance for two traits; ***- best performance for three traits

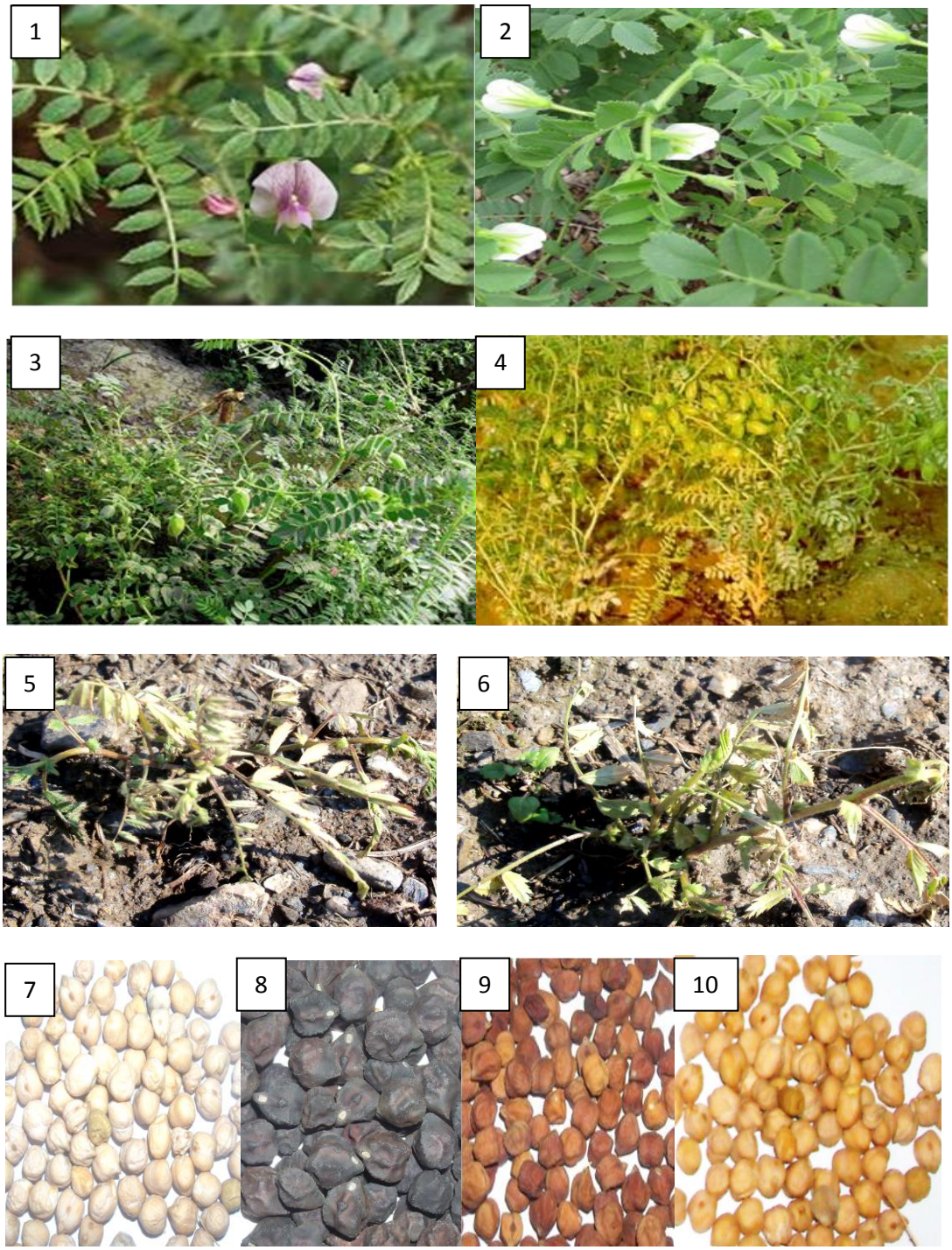


Figure 4.7: Plates 1-4 representing flowering to pods maturity stage for estimation of genetic diversity, 5-6 showing wilted chickpea plants in experimental field and 7-10 representing different size and color of seeds in cultivated chickpea

4.4 Correlation studies

The correlation coefficient among quantitative traits was computed for three years (2009, 2010 and 2011) and showed that 100/seed weight was highly positively correlated with grain yield (r = 0.36a, r = 0.21c) and significantly correlated with the same trait (r = 0.57b) and total biomass (r = 0.56a and 0.65b), where showed this relation highly significant as (r = 0.39c). Similarly grain yield showed a positive significant correlation with total biomass (r = 0.67a, 0.33b and 0.45c) and harvest index (r = 0.46a, 0.33b and 0.68c), while total biomass was negatively correlated to harvest index. The three years data were pooled and presented in Table 4.5 and also graphically presented in Figures 4.8a-i.

Table 4. 5: Correlation coefficient among quantitative traits harvested during 2008-2009, 2009-2010 and 2010-2011 reported in chickpea germplasm

	100/seed weight	Grain yield	Total biomass
Grain yield	0.36* a *0.57*** *b* 0.21* c		
Total biomass	0.56** a *0.65*** *b* 0.39* c	0.67** a *0.33** b* 0.45* c	
Harvest index	- 0.03 a *0.01 b* -0.07 c	0.46** a *0.33** b* 0.68** c	- 0.21 a *- 0.32 b* -0.19 c

a-2008- 2009, b- 2009- 2010, c- 2010-2011, $P \leq 0.01$, highly significantly correlated
$P \leq 0.05$, significantly correlated

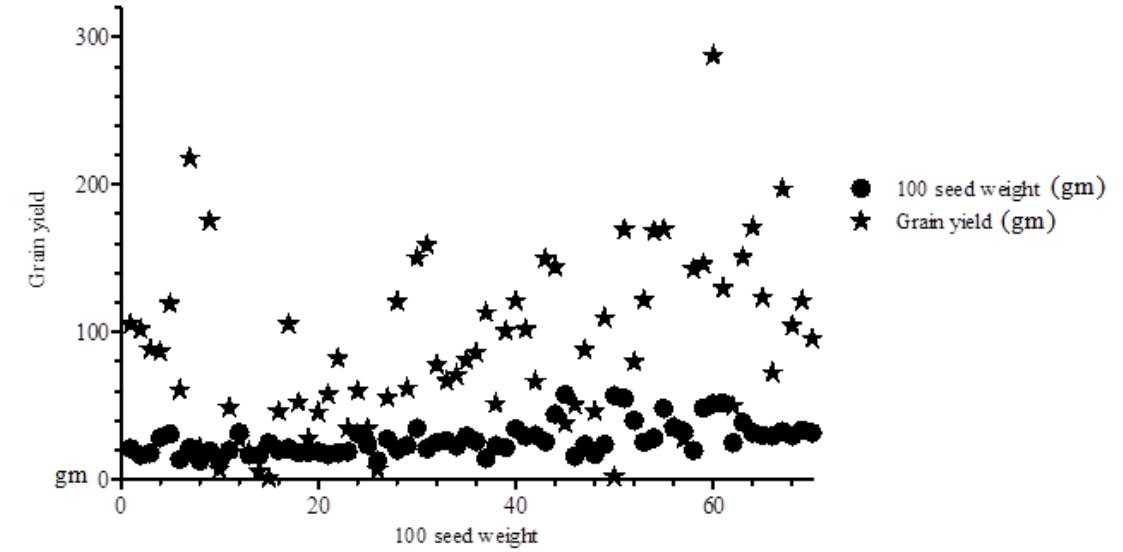


Figure 4.8a:Correlation coefficient between grain yield and 100 seed weight (2008-2009)

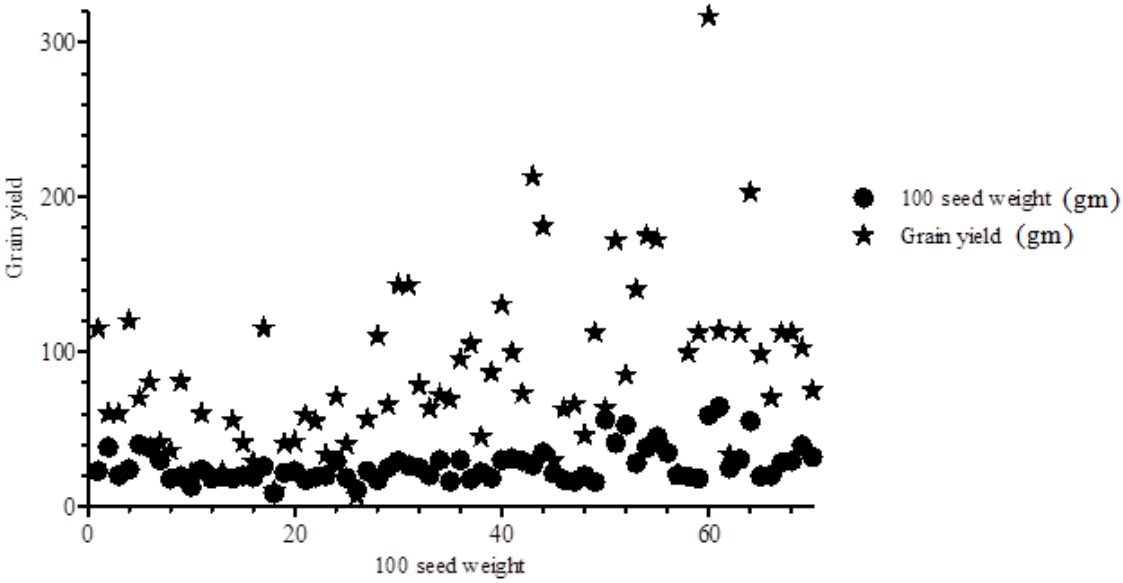


Figure 4.8b:Correlation coefficient between grain yield and 100 seed weight (2009-2010)

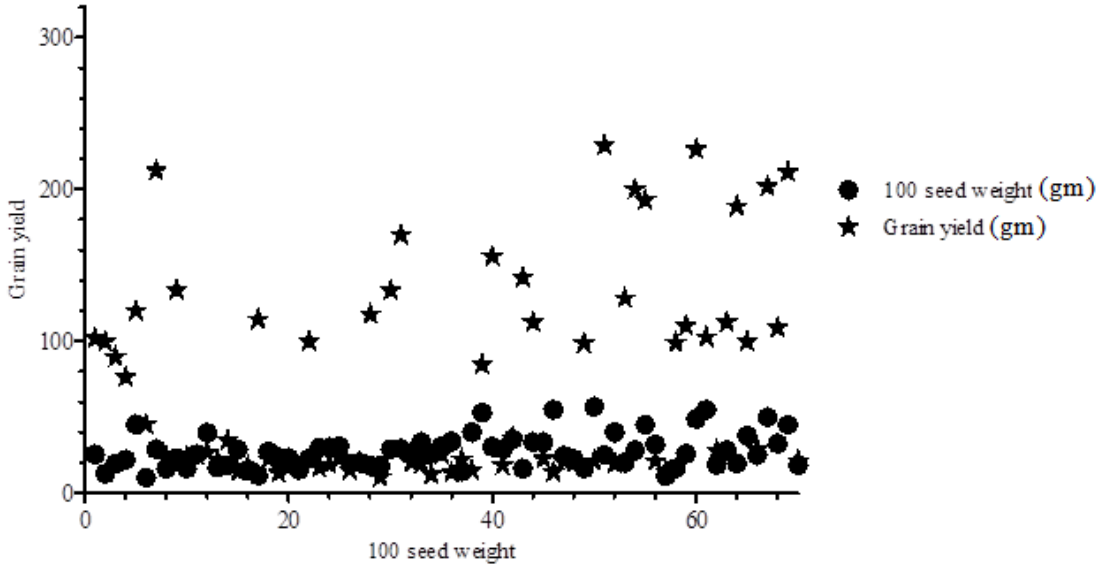


Figure 4.8c: Correlation coefficient between grain yield and 100 seed weight (2010-2011)

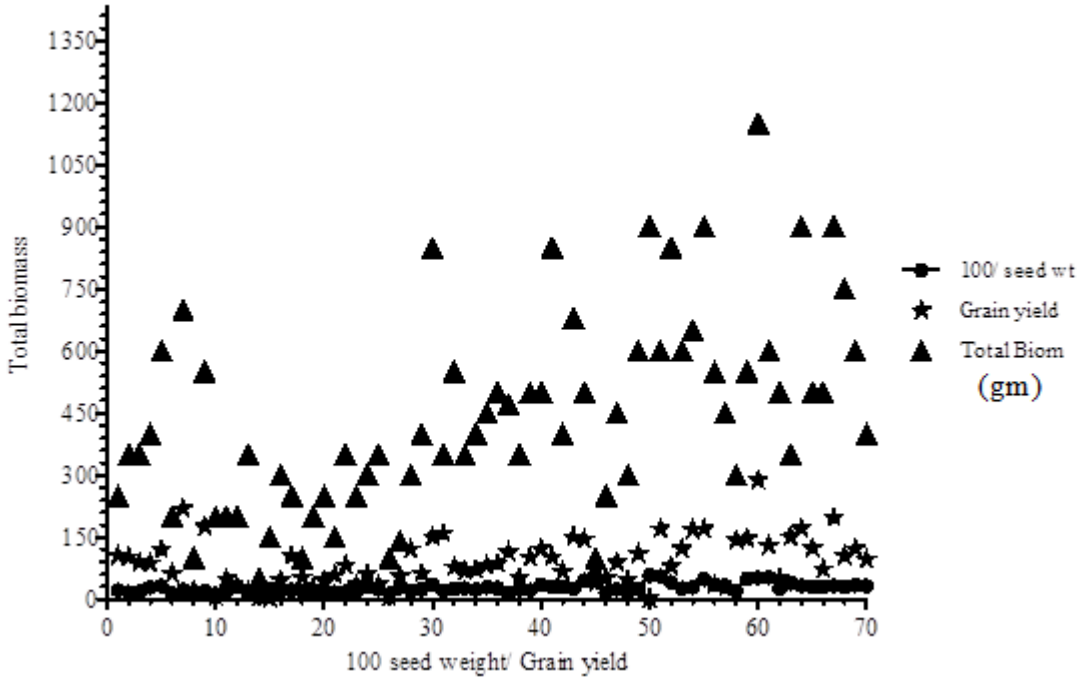


Figure 4.8d: Correlation coefficient between total biomass, grain yield and 100 seed weight (2008-2009) in chickpea

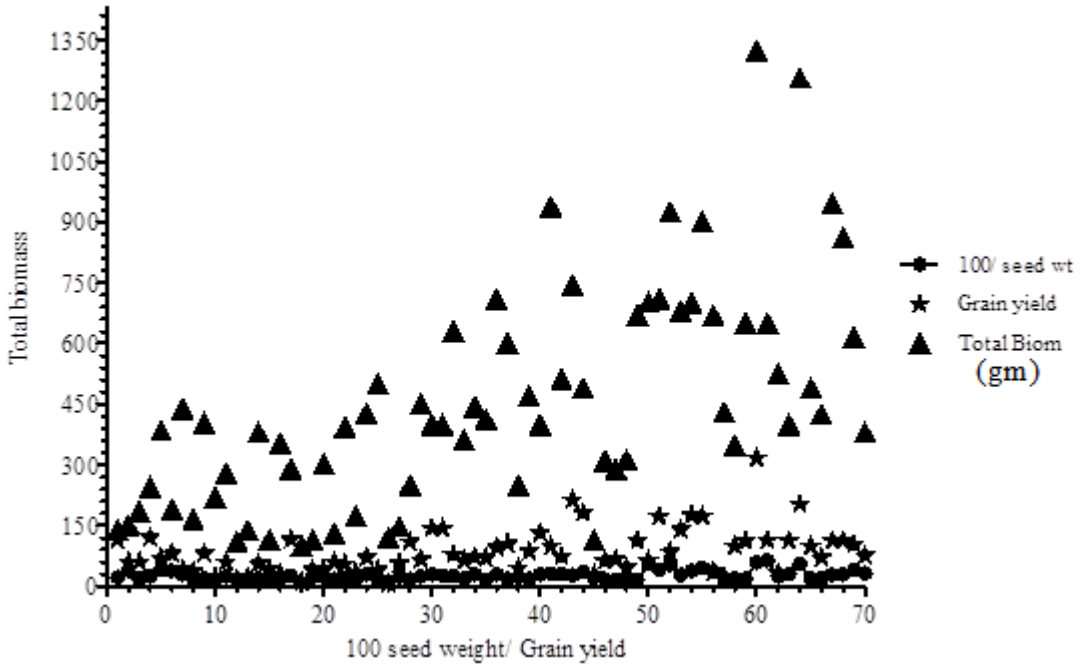


Figure 4.8e: Correlation coefficient between total biomass, grain yield and 100 seed weight (2009-2010) in chickpea

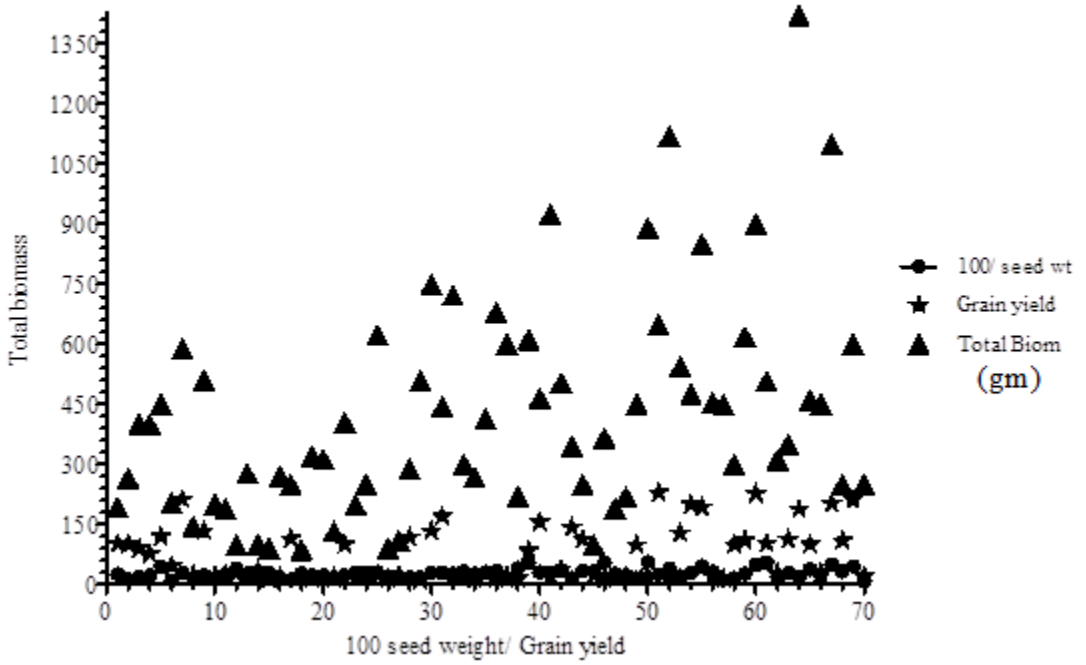


Figure 4.8f: Correlation coefficient between total biomass, grain yield and 100 seed weight (2010-2011) in chickpea

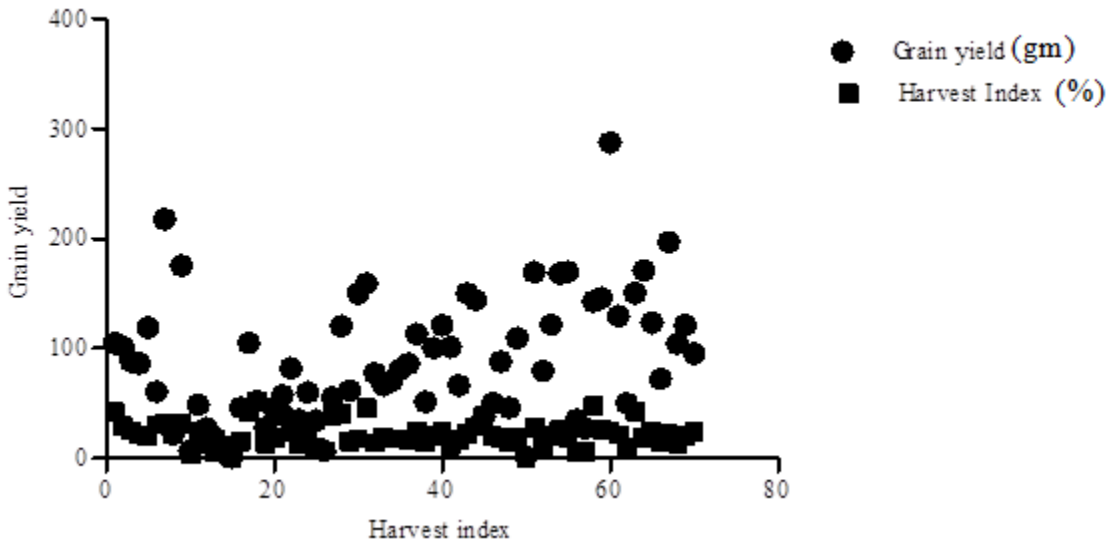


Figure 4.8g: Correlation coefficient between grain yield and harvest index (2008-2009) in chickpea

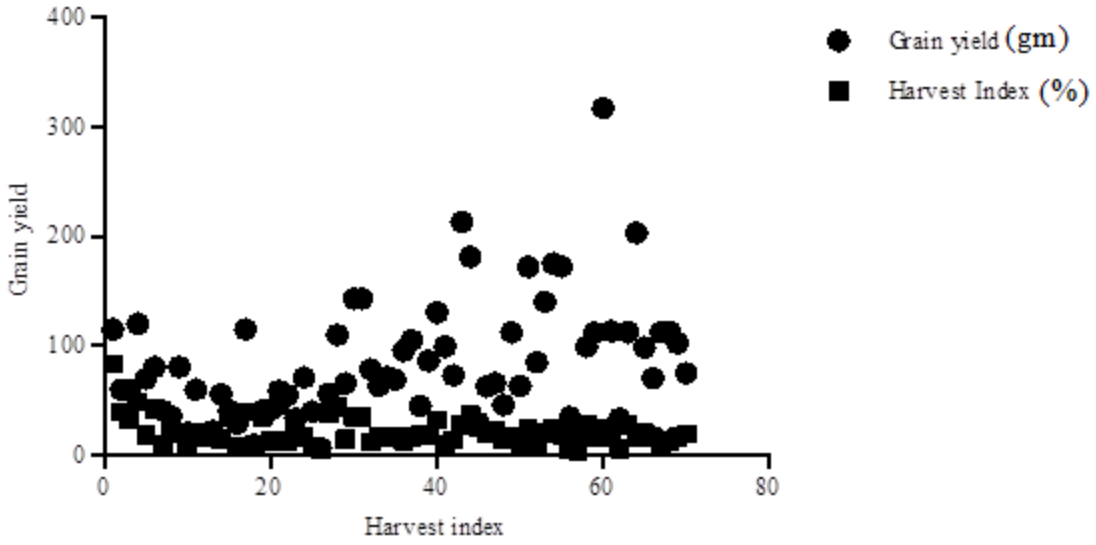


Figure 4.8h: Correlation coefficient between grain yield and harvest index (2009-2010) in chickpea

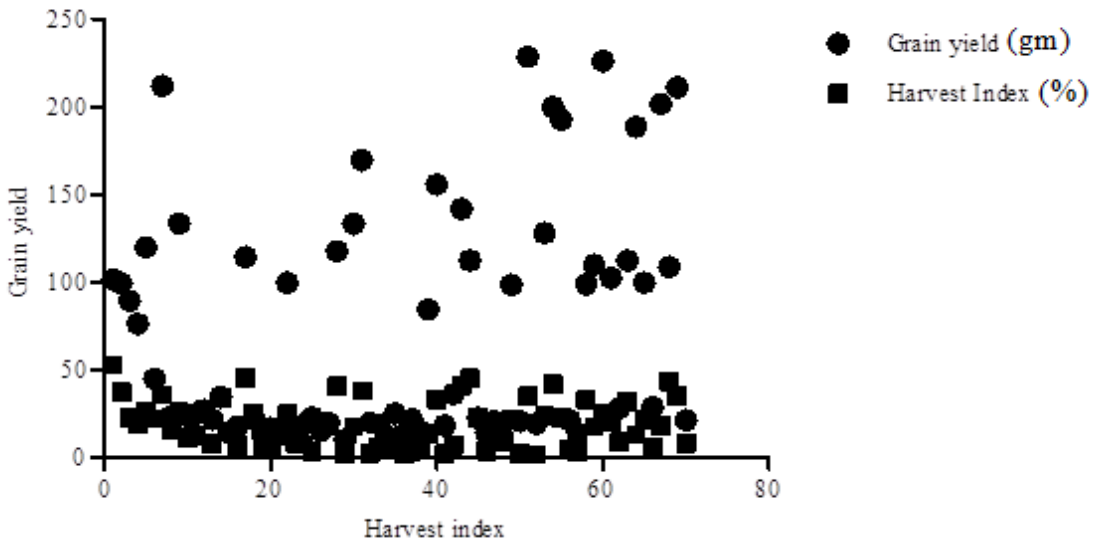


Figure 4.8i: Correlation coefficient between grain yield and harvest index (2010-2011) in chickpea

4.5 Frequency distribution of seed size categories

To check the influence of seed size upon seed weight the total germplasm was divided into groups based on seed size of each accession (Table 4.6). It was observed that out of 70 accessions, 40% had seed size range from 3-4 mm and categorized into small seed size germplasm. While, 44% were ranged from 4.2 – 7.2mm and 16% ranged in 8-9.9mm seed size, they were categorized into medium and large size seeds respectively. When the cumulative frequency was calculated, 84% accessions were examined with small (3-4mm) and medium (4.2- 7.2mm) size (Table 4.7, figure 4.9). It is evident from table 4.7 that medium and large size chickpea accessions have attained maximum 100 seed weight, ranged from 30- 57gm. The accessions 3037, 3040, 3065, 3027, 3063, 3059, 3058, 3021, 3023, 3020 and 2654 all of USA origin were observed of medium size with value of 100 seed weight calculated as 56.66, 44.27, 39.37, 37.65, 37.11, 35.51, 32.53, 31.77, 31.72, 31.12 and 31.15gm respectively. On the other hand the accessions 3056, 3054, 3043, 3026, 3047 and 3064 were of large size and USA origin have attained the maximum mean value of 100 seed weight scored as 57.18, 53.16, 46.19, 37.63, 30.92 and 30.71gm respectively. Wherease, the accession 2562 of Pakistani origin among the selected medium and large size seeds were fell into the large size seed caregory with a

maen value of 100 seed weight 30.33gm (4.8).

Table 4.6: Seed size distribution based on length and width of chickpea accessions

S/ No	Seed size (mm)	Seed class distribution	Representative accessions	Country of origin
1	3- 4	Small	1898, 1936, 1998, 2023, 2188, 2237, 2272, 2273, 2278, 2532 2544.	Pakistan
			2595, 2611, 2616, 2629, 2650 2831, 3011, 3015, 3016 3033 3035, 3041, 3057.	USA
2	4.2- 7.2	Medium	1995, 2234, 2235, 2236, 2430 2441, 2473, 2497, 2499, 2531 2553	Pakistan
			2654, 2819, 2859, 3017, 3020 3021,3023,3024, 3027 3031 3032, 3037, 3039, 3040, 3042 3044, 3045,3046 3058, 3059 3062, 3063, 3065, 3066.	USA
3	8 ≥ 9.9	Large	2558, 2562	Pakistan
			2855, 3022, 3026, 3043, 3047 3054, 3056, 3061 3064.	USA

Table 4.7: The frequencies and cumulative frequencies of seed size categories in chickpea

S/ No.	Seed size distribution	Frequency (%)	Cumulative frequency	Range of seed size
1	Small	40	40	3 – 4 mm
2	Medium	44	84**	4.2 - 7.2 mm
3	Large	16	100	8 – 9.9 mm

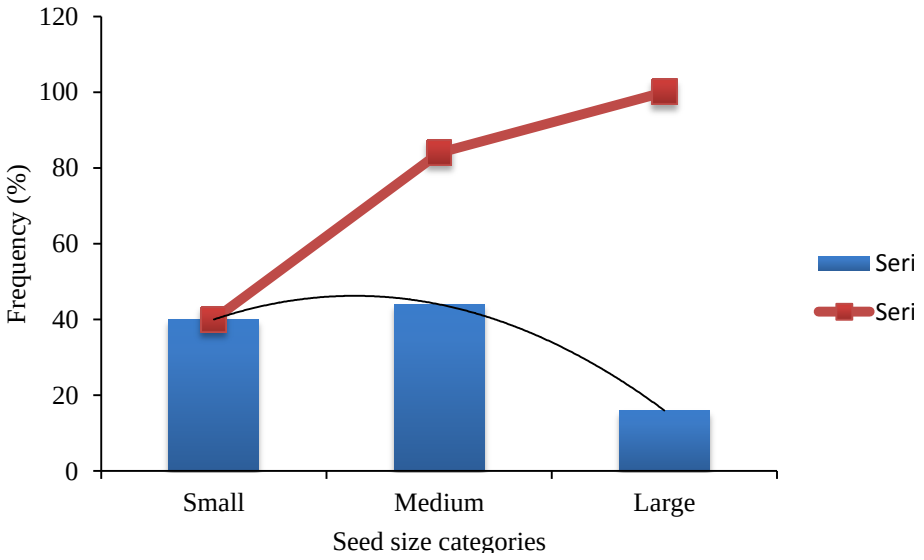


Figure 4.9: A comparative graph between frequency (%) and cumulative frequency of seed size grouping in chickpea local and exotic accessions. Series-1= frequency (%), series-2= cumulative frequency (%); the running total of frequencies showed the future trend of seed size distribution in chickpea

Table 4.8: The accessions of medium and larger size with maximum 100-seed weight

Serial No.	Accession No.	Origin	100 seed weight (gm) (Mean value)	Seed size (mm)	Category
1	3037	USA	56.66	4.2	Medium
2	3040	USA	44.27333	4.2	Medium
3	3065	USA	39.37333	7.2	Medium
4	3027	USA	37.65667	4.2	Medium
5	3063	USA	37.11333	7.2	Medium
6	3059	USA	35.51667	7.2	Medium
7	3058	USA	32.53333	7.2	Medium
8	3021	USA	31.77667	7.2	Medium
9	3023	USA	31.72667	7.2	Medium
10	3020	USA	31.12333	7.2	Medium
11	2654	USA	31.15	4.2	Medium
12	3056	USA	57.18333	9.9	Large
13	3054	USA	53.16333	9.9	Large
14	3043	USA	46.19333	9.9	Large
15	3026	USA	37.63333	9.9	Large
16	3047	USA	30.92	8.0	Large
17	3064	USA	30.71	8.0	Large
18	2562	Pakistan	30.33333	8.0	Large
19	3022	USA	29.81667	8.0	Large
20	3061	USA	29.26	9.9	Large

4.6 Screening of chickpea for wilt resistance

4.6.1 Screening at seedling and reproductive stage

During 2012, a significant variation in accessions was obtained in response to *Fusarium* wilt (Table 4.9). Following the disease rating scale described by Iqbal *et al*. (2005), the accessions were grouped into four categories (Figure 4.10). The disease response of chickpea accessions at two growth stages is given (Appendix 12 and 13). It was observed that 20 accessions *i.e.,* 1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2562, 2595, 2611, 3037, 3039, 3043, 3054, 3056, 2819, 2831, 3059, 2855 were highly resistant, 20 (2272, 2273, 2473, 2499, 2531, 2558, 2532, 2654, 3011, 3020, 3021, 3023, 3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063) were resistant, 9 (1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061) were moderately resistant or tolerant, while the rest 21 (3027, 3031, 3033, 3040, 3044, 3047, 2629, 2650, 2859, 3062, 3064, 3022, 3017, 3016, 2616, 2544, 2234, 1936, 2237, 2278, 2497) accessions were susceptible at seedling stage in the field.

In case of screening in the field sick-bed, at reproductive stage, 14 accessions (1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 2611, 3043, 3054, 3059, 2855) were found highly resistant, 17 (2272, 2273, 2473, 2531, 2654, 3011, 2532, 3020, 3021, 3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063) were resistant, 9 (1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061) were tolerant and 30 accessions (3027, 3031, 3033, 3040, 3044, 3047, 2629, 2650, 2859, 3062, 3064, 2544, 2234, 1936, 2237, 2278, 2497, 3022, 3017, 3016, 2616, 3023, 2499, 2558, 3039, 3056, 2831, 2819, 3037, 2562) were found susceptible to wilt disease (Figure 4.10, Appendix 12 and 13). However, disease incidence (%) was calculated to be 30% at seedling stage and reached up to 42.85% at reproductive stage.

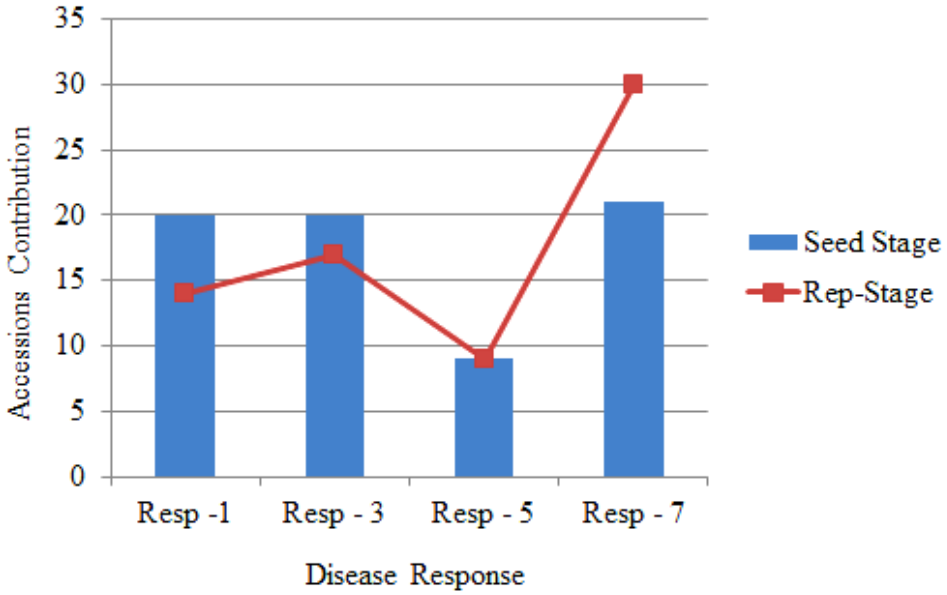


Figure 4.10: Classification of chickpea accessions with respect to their wilt response in field at seedling and reproductive stage (Resp = Response to disease, Rep = Reproductive to pods maturity stage, 1= Highly resistant (HR); 3 = Resistant (R); 5 = Moderately resistant (MR); 7 = susceptible

Table 4.9: *t*- test for *Fusarium* wilt response of chickpea local and exotic lines in field

SOV	t-value	df	Mean	Mean df	SE	SD	CI: 95% Lowr	CI: 95% upper	p-value
Seedling stage	6.155	3	17.50	17.5	2.843	5.686	8.452	26.55	0.01
Reproductive/pods maturity stage	3.905	3	17.50	17.5	4.481	8.963	3.238	31.76	0.01

alpha ≤ 0.050, df = difference, SE.= standard error, SD= standard Deviation, CI-Confidence Interval

In 2013, the chickpea accessions screened for *Fusarium* wilt under artificial disease conditions in greenhouse showed a wide range of genetic variation (Table 4.10). The disease response of chickpea lines at two growth stages is given in appendix 14 and 15. At seedling stage, 18 accessions (1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 2611, 3037, 3039, 3043, 3054, 3056, 2819, 3059, 2855) were found highly resistant, 32 (2272, 2273, 2473, 2499, 2531, 2558, 2654, 3011, 2532, 3020, 3021, 3023,

3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063, 1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061, 3040, 2831, 2562) were resistant, 14 (3047, 3022, 1936, 2859, 3062, 3064, 2544, 3017, 3016, 2616, 2237, 3031, 3033, 3044) were moderately resistant or tolerant and 6 (3027, 2629, 2650, 2234, 2278, 2497) were susceptible. While, at reproductive stage 15 (2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 3039, 3043, 3054, 3056, 2819, 3059, 2855) accessions were highly resistant, 26 (2272, 2273, 2473, 2499, 2558, 2654, 3011, 2532, 3020, 3021, 3023, 3035, 3045, 3046, 3057, 3065, 3066, 3063, 1995, , 3015, 3032, 3042, 3024, 3058, 3061, 3040) resistant, 12 (3047, 3022, 1936, 2859, 3062, 3064, 2544, 3017, 3016, 2616, , 3033, 3044) were tolerant and 17 (1898, 1998, 2611, 3027, 2629, 2650, 2234, 2278, 2497, 3037, 2562, 2531, 3026, 2831, 3041, 2237, 3031) were found susceptible to the disease (Figure 4.10a). The disease incidence ranged from 0% to 8.57% at seedling stage and reached up to 24.28% at reproductive stage.

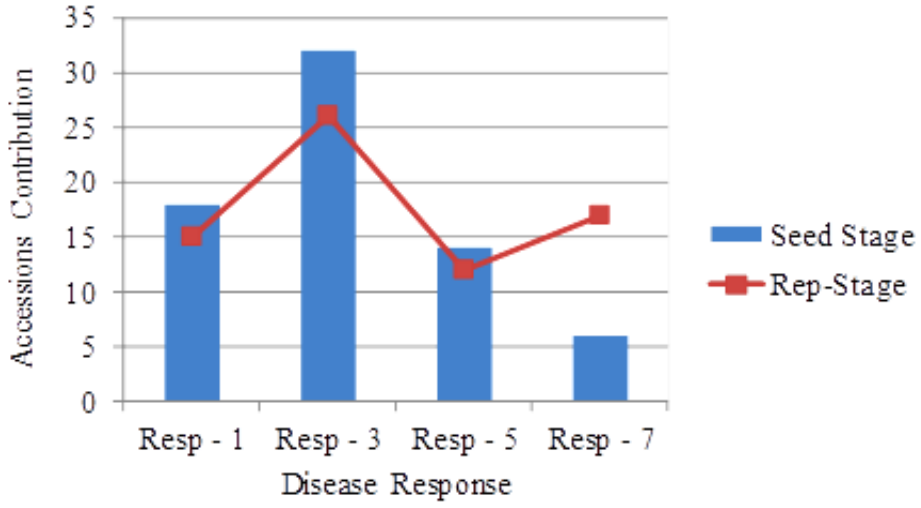


Figure 4.10a: Classification of chickpea accessions with respect to their wilt response in greenhouse conditions at seedling and reproductive stage (Resp = Response to disease, Rep = Reproductive to pods maturity stage, 1= Highly resistant (HR); 3 = Resistant (R); 5 = Moderately resistant (MR); 7 = susceptible.

Table 4.10: *t*- test for *Fusarium* wilt response of chickpea local and exotic lines in greenhouse conditions

SOV	t-value	df	Mean	Mean df	SE	SD	CI: 95% Lowr	CI: 95% upper	p-value
Seedling stage	3.217	3	17.50	17.5	5.439	10.88	0.190	34.81	0.01
Reproductive/pods maturity stage	5.807	3	17.50	17.5	3.014	6.028	7.909	27.09	0.01

alpha ≤ 0.050, df = difference, SE.= standard error, SD= standard Deviation, CI-Confidence Interval

4.7 Biochemical analysis

4.7.1 SDS-PAGE

The extracted seed storage proteins when run on gel revealed a total of 16 high molecular weight polypeptide bands were scored; the position of each band was tagged through an arrow (Figure 4.11, 4.12a- 4.12d, appendix 16). The low molecular weight bands were not reproducible, therefore, these were not considered in the study.

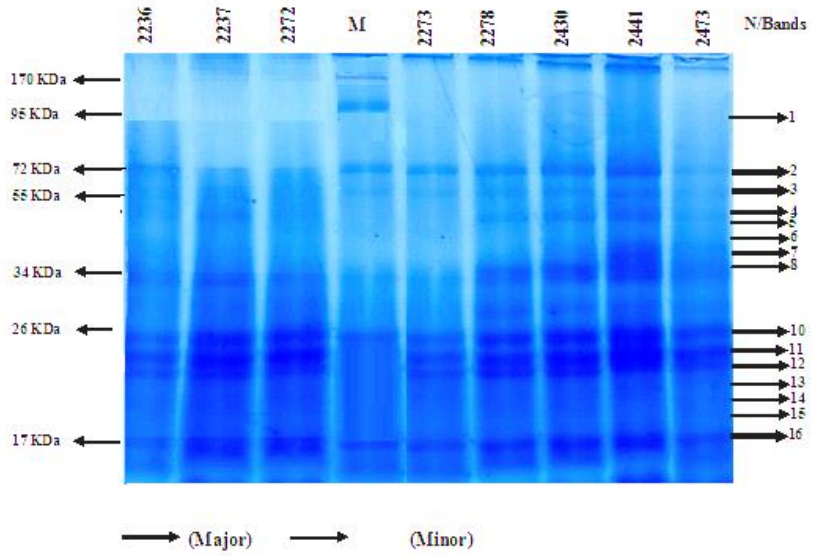
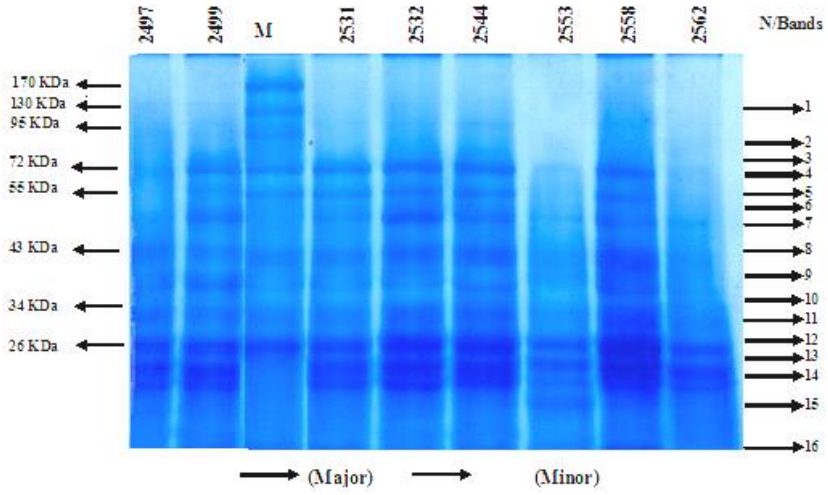
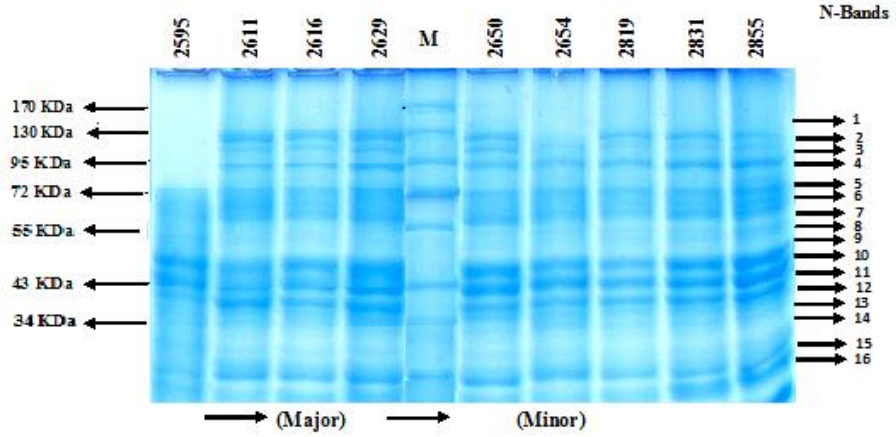


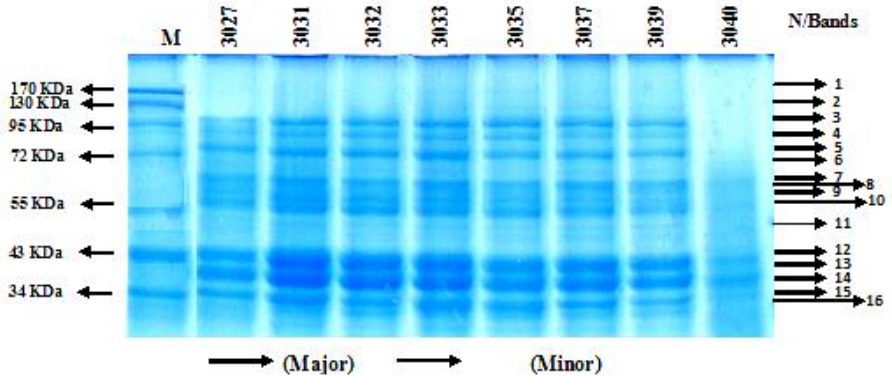
Figure 4.11: Electrophorograms showing the distribution of different molecular weight protein in chickpea accessions. Arrows indicate the presence of band, KDa-K-Daltons.



(a)

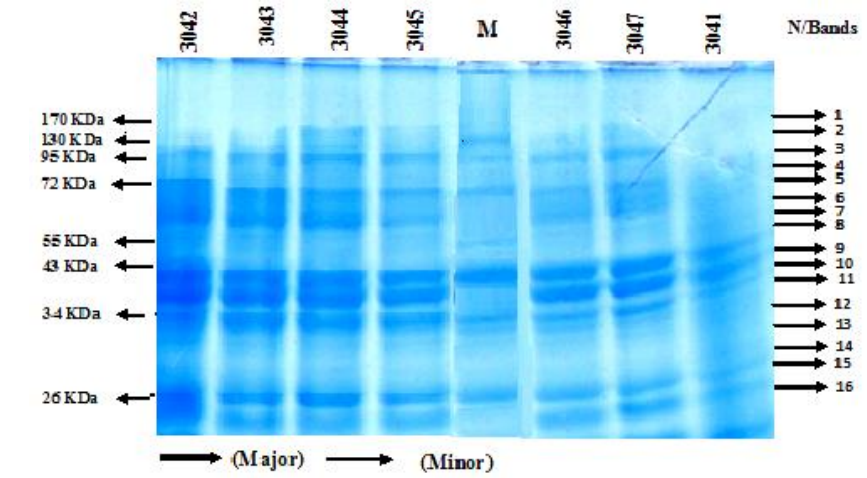


(b)

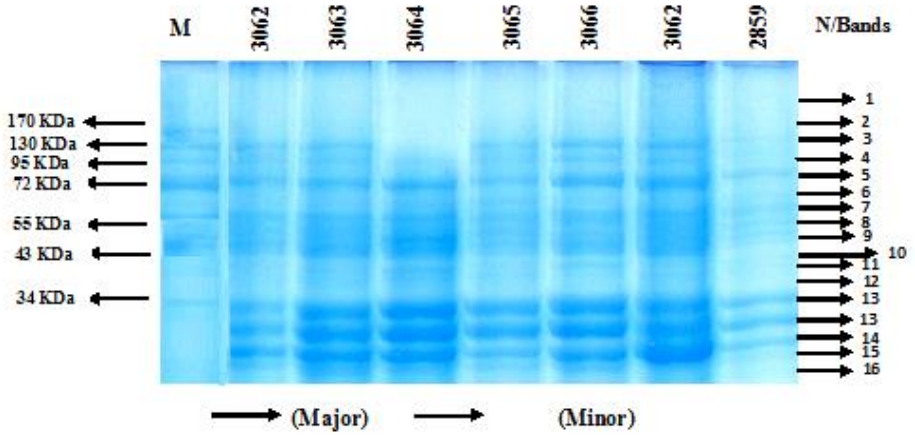


(c)

Figure 4.12a,b,c: Electrophorograms showing the distribution of different molecular weight protein in chickpea accessions. Arrows indicate the presence of band, KDa-K-Daltons.



(d)



(e)

Figure 4.12 d,e: Electrophorograms showing the distribution of different molecular weight protein in chickpea accessions. Arrows indicate the presence of band, KDa-K-Daltons.

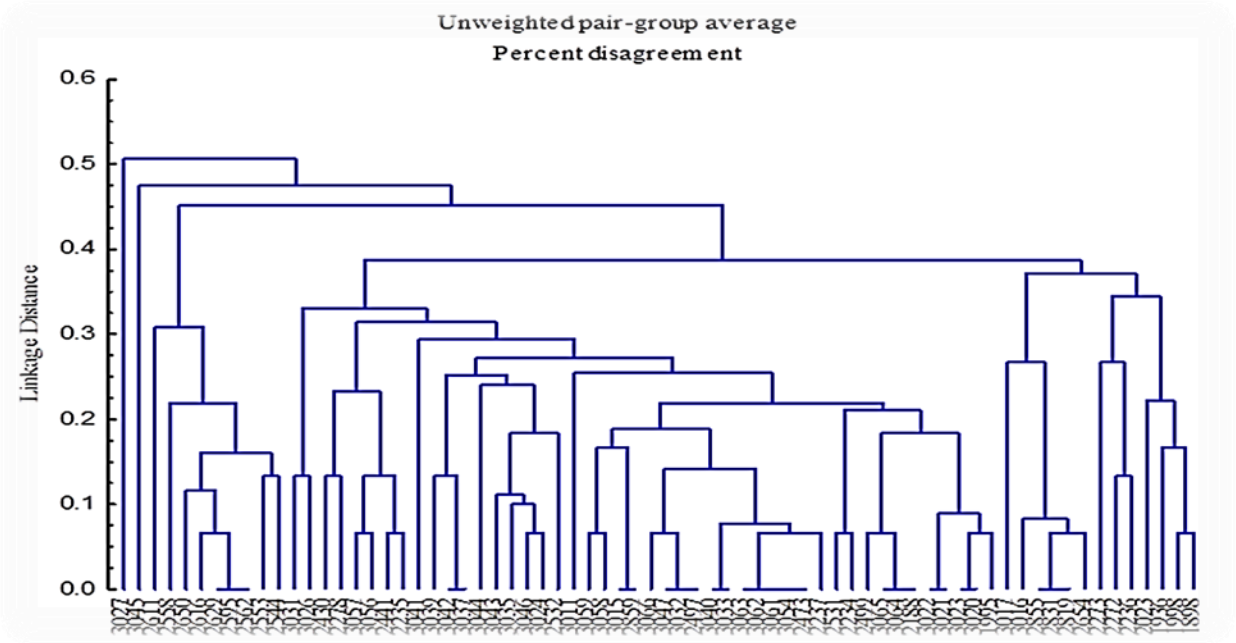


Figure 4.13: Cluster analysis of chickpea 70 genotypes using SDS- PAGE

The cluster analysis of 70 accessions estimated 50% genetic diversity. The dendrogram was constructed on the basis of un-weighted pair- group average percent disagreement (UPGMA) divided the total germplasm into two major lineages: L-1 and L-2 at a linkage distance 0.5. Lineage first reported 3027 and 3045 of USA were remained unresolved. Contrary to this lineage second further splitted into three clusters at a linkage distance measured 0.375 (Figure 4.13). Cluster-1 comprised 5 USA, 2611, 2650, 2616, 2629, 2595 and 4 Pakistani, 2558, 2562, 2553 and 2544 accessions. Cluster-2 was observed the largest group with 33 USA accessions mentioned as 3031, 3026, 3041, 3057, 3056, 3039, 3042, 3037, 3044, 3043, 3035, 3046, 3024, 3011, 3059, 3058, 3015, 3066, 3047, 3032, 3040, 3033, 3063, 3062, 3061, 3054, 2859, 3065, 3064, 3022, 3021, 3023, 3024 and 13 accessions, 2430, 2278, 2441, 2235, 2532, 2497, 2473, 2237, 2531, 2234, 2499, 2188 and 1995 of Pakistani origin. In addition, cluster-3 was consisted of 6 USA accessions given as 3017, 3016, 2855, 2831, 2819, 2654 and 7 Pakistani accessions were coded as 2273, 2272, 2236, 2023, 1936, 1998 and 1898 were grouped as 13.2%, 67.6% and 19.1% of the total population respectively. However, the accessions 2629, 2595, 2562, 3042, 3037, 3015, 2859, 3047, 3032, 2497, 3040, 3033, 3063, 3062, 3061, 3054, 2473, 2855, 2831 and 2819 showed 100% similarity in their protein banding outline distributed among three clusters and constituting about 28% of the total germplasm evaluated (Table 4.11).

Table 4.11: Cluster analysis based on disagreement using SDS-PAGE in chickpea

Clusters	Genotype
C- 1	2611, 2650, 2616, 2629, 2595 (USA), 2558, 2562, 2553, 2544 (Pakistan)
C- 2	3026, 3031, 3041, 3056, 3057, 3039, 3037, 3042, 3044, 3035, 3043, 3046, 3011, 3024, 3059, 3015, 3058, 3066, 3032, 3047, 3040, 3063, 3033, 3062, 3054, 3061, 3065, 2859, 3064, 3021, 3022, 3023, 3024 (USA) 2430, 2278, 2441, 2235, 2532, 2497, 2473, 2237, 2531, 2234, 2499, 2188, 1995 (Pakistan)
C- 3	3017, 3016, 2855, 2831, 2819, 2654 (USA), 2273, 2272, 2236, 2023, 1936, 1998, 1898 (Pakistan)
Unresolved	3027, 3045

4.8 Molecular characterization

4.8.1 Genetic diversity using RAPD markers

The accessions were tested through twenty RAPD makers for estimation of genetic diversity in the collected lines based on loci presence and absence. Out of which five RAPD makers were polymorphic, while the remaining markers were not considered for further analysis due to their poor amplification, reproducibility and mono-morphic nature. A total of nine bands have been scored by using OPA4, six by OPA9, sixteen by OPG13 and seventeen for each UBC181 and UBC733b (Figures 4.14a - 4.14e, appendix 20- 24) Among RAPD makers, UBC733b, UBC181 showed 89.80 and 77.43% allele polymorphism respectively. While, OPA4, OPA9 resulted in 36.18% and 27.94% polymorphism or genetic diversity. The primer OPG13 however expressed 17.27% polymorphism in banding profile (Table 4.12).

Table 4.12: Sequences of the RAPD primers used for molecular analysis of chickpea

S/No	Primer	sequence	Bands	Mean	Std.Dev.	*t*-value	Coef.Var.
1	UBC181	ATGACGACGG	17	1.26	0.97	10.81	77.43**
2	UBC733b	GGGAAGGGAG	17	1.11	1.00	9.32	89.80**
3	OPA4	AATCGGGCTG	9	1.77	0.64	23.12	36.18
4	OPA9	GGGTAACGCC	6	1.86	0.52	29.95	27.94
5	OPG13	CTCTCCGCCA	16	1.94	0.34	48.44	17.27

St.Dev-standard deviation: Coef. Vr- Coefficient of variation

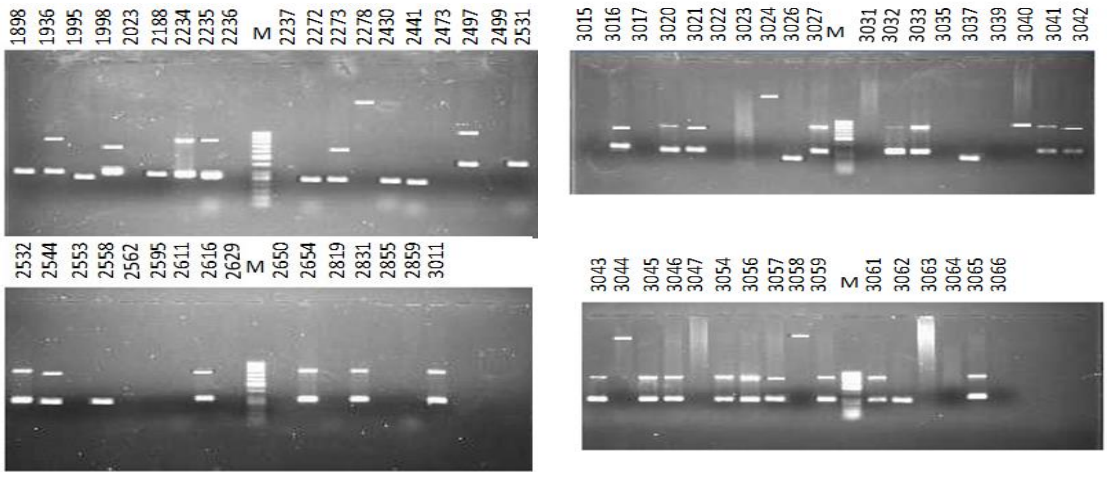


Figure 4.14a: PCR amplification profile of genomic DNA from chickpea local and exotic accessions using RAPD primer OPA4

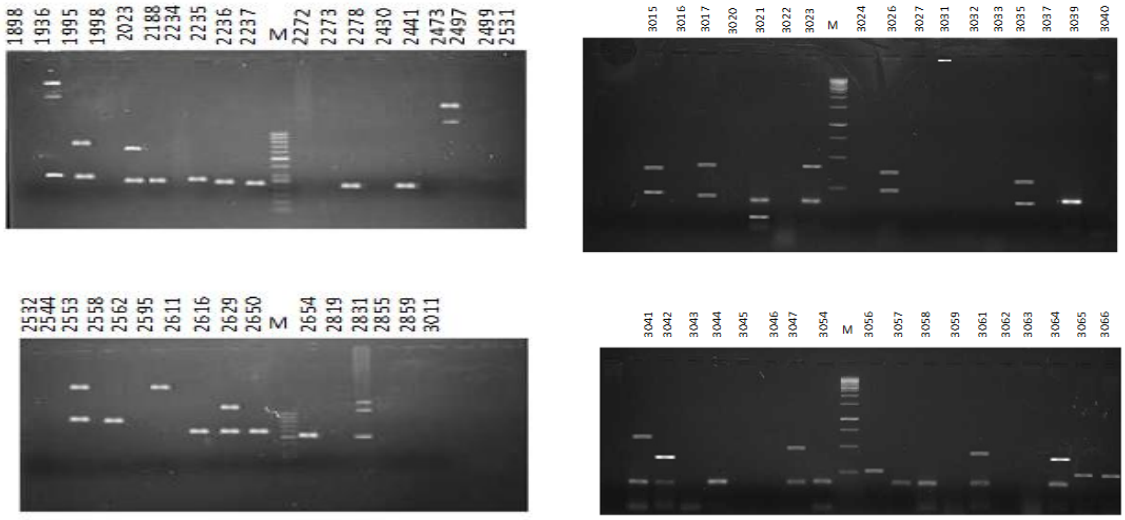


Figure 4.14b: PCR amplification profile of genomic DNA from chickpea local and exotic accessions using RAPD primer OPA9

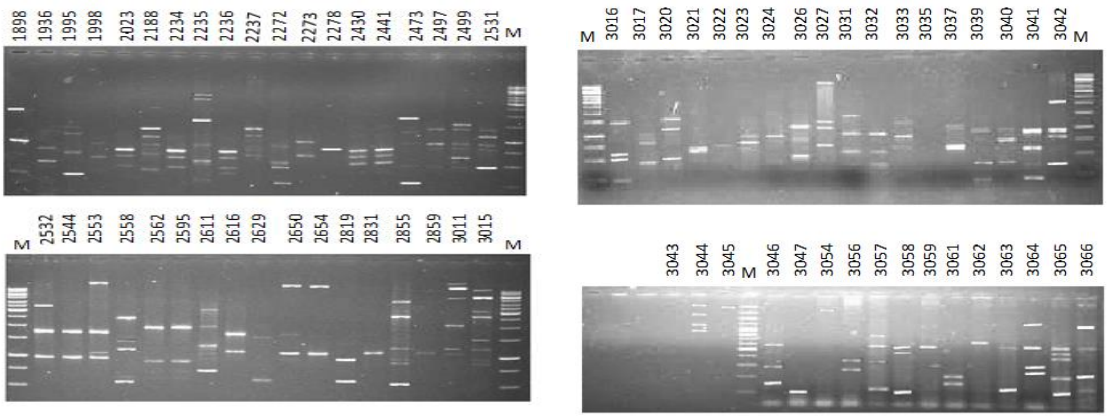


Figure 4.14c: PCR amplification profile of genomic DNA from chickpea local and exotic accessions using RAPD primer OPG13

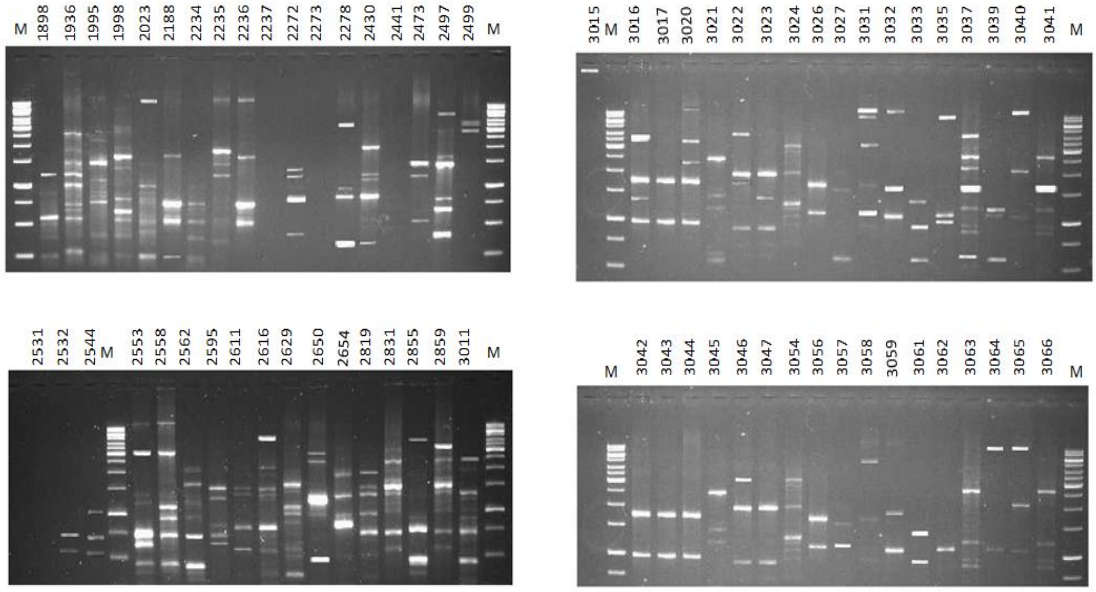


Figure 4.14d: PCR amplification profile of genomic DNA from chickpea local and exotic accessions using RAPD primer UBC181

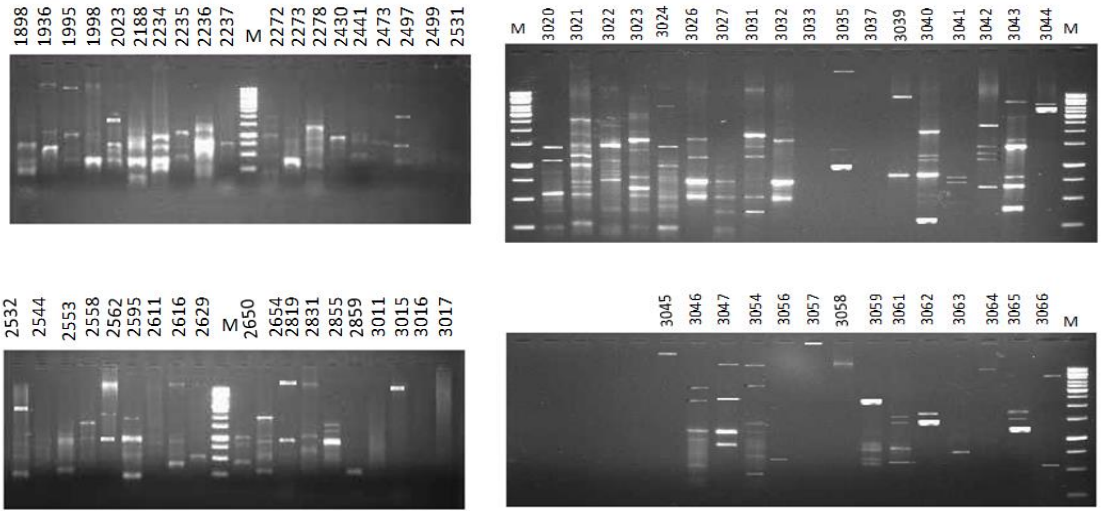


Figure 4.14e: PCR amplification profile of genomic DNA from chickpea local and exotic accessions using RAPD primer UBC733b

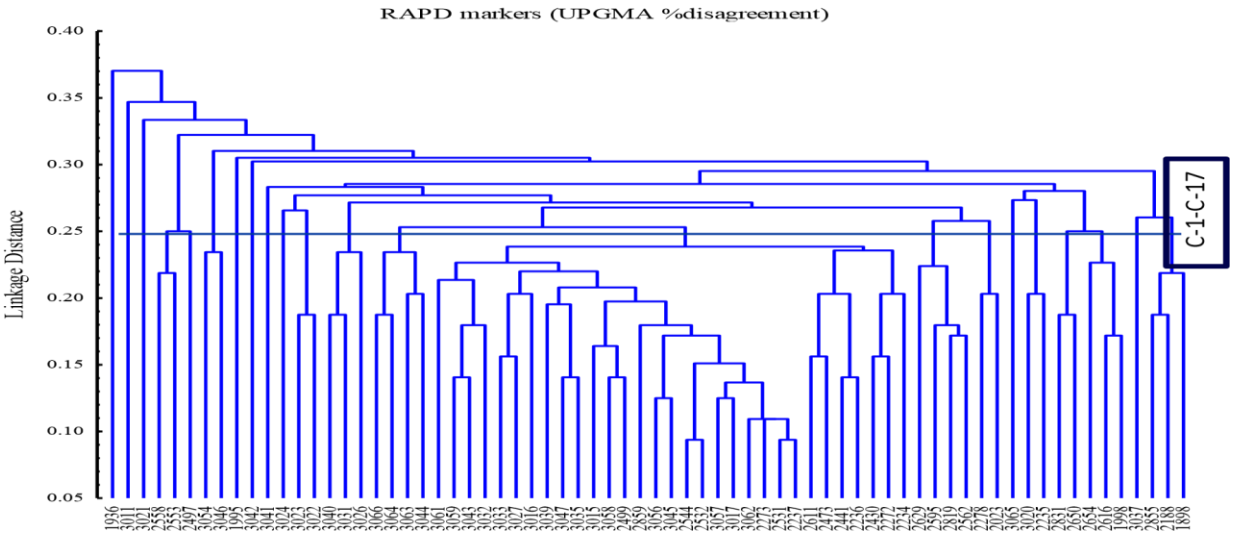


Figure 4.15: Dendrogram of 70 chickpea accessions based on RAPD data using UPGMA

At 0.25 linkage distance the dendrogram based on RAPD markers was divided into seventeen clusters for 70 chickpea accessions to estimate the genetic diversity (Figure 4.15). The analysis sorted the total accessions into two main groups, which were further divided into 17 clusters. Cluster-12a grouped maximum number of accessions contributing 34.2% of the total germplasm indicated highly polymorphic bands at different linkage distances. Thus comprised the promising lines 3059 and 3043 at a same linkage distance 0.14, while 3039, 3063 at 0.20 and 3056 at 0.13 linkage distance are closely allied with each other. While Cluster-1, 2 and 3 was composed of 1936 at a linkage distance 0.37, 3011 at 0.35 and 3021 at a linkage distance 0.33 respectively. Cluster-4 grouped together 2558, 2553 reported 22% dissimilarity which was 25% in 2497. The accessions 2544, 2532, 2531 and 2237 of Cluster-12a occurred at a linkage distance 0.09 similar with 3062 and 2273 were found 11% varied in their banding outline made on the basis of presence and absence of allele. The accessions 3059, 3043, 3047, 3935, 3958, 2499 (C- 12a) and 2441, 2236 (C- 12b) however were 14% dissimilar at a same linkage distance 0.14. The accessions 3033, 3027 (C- 12a); 2611, 2473, 2430 and 2272 (C- 12b) revealed 16% variation located at a distance 0.16. On the other hand 3015 (C-12a), found at a linkage distance 0.17 followed the accession 3024 (C- 9) and 3065 (C- 14) having 29% diversity and found at 0.29 linkage distance revealed high degree of polymorphism regarding their banding pattern as compared to other accessions. Similarly 3022 and 3040 occupied cluster-10 and 11 at similar linkage distance 0.19. The genotype 3054 of cluster-5 and 3037 showed slight difference in their banding outline represented 23-25% dissimilarity (Figure 4.9.9- 4.9.18). Cluster-16, 13 and cluster-17 was comprised of 2654, 2629 and 3037 at the same linkage distance 0.23 and the degree of similarity among them found 77% subjected 23% genetic variability when compared with other accessions (Figure 4.15).

4.8.2 Genetic diversity using SSR markers

The accessions were also tested through twenty SSR makers to determine genetic diversity in seventy lines based on loci presence and absence. Out of which fifteen SSR makers were polymorphic (Figure 4.16a- 4.16m, appendix 25). Thus among the SSR markers CaSTMS15, CaSTMS2, TA194 and TA71 indicated 97.88%, 82.24%, 71.19%

and 70.46% allele polymorphism respectively regarding genetic variability among the accessions. While it has been calculated 63.7% for each TA130, TR1 and 65.94% for CaSTMS21. The SSR markers TA72 and TR29 however scored the same allele polymorphism (%), *i.e.,* 21.31%. Similarly TA200, TA135 and TA22 represented 43-45% genetic variability when estimated. The remaining markers TA46 and TR130 have also shown similar variation in alleles ranged from 50- 52% respectively. The SSR marker TR7 showed 36.18% allele polymorphism to determined genetic variation among the accessions (Table 4.13).

The dendrogram formed by UPGMA percent disagreement divided the total germplasm into two lineages, L- I and L- II at a linkage distance 0.55, thus revealing 55% genetic diversity (Figure 4.17). The L- I consist up of cluster-1 including the accessions 3011 and 2859 at distinct linkage distance 0.4. The lineage- II however was further splitted into 7 clusters. In which cluster-2 comprised three accessions 3061, 3662 showed linkage distance 0.275 and genetically highly varied accession 1898 at linkage distance measured as 0.4. Cluster-3 represented the accessions 3045, 3016, 2237, 3059, 2234 with similar banding profile at linkage distance 0.125 and 3041, 3035, 3027 with 2273 at 0.075. While 2499 appeared at linkage distance 0.275 revealed variations from other accessions of the same group. Similarly cluster-3 also grouped the accessions 3033, 3023, 2430, 2497 and 3016 at various linkage distances ranged from 0.175- 0.225. In case of cluster-4 there were six accessions observed *i.e.,* 2631, 2611 at linkage distance 0.125 and 2819, 2553 again at 0.075 linkage distance. The cluster-4 also included 3015 and 2532 at a linkage distance ranged from 0.2- 0.275. Cluster-5 having only three accessions 3047, 3064 showing linkage distance 0.125 and 2473 at 0.2 linkage distance. Cluster-7 grouped the diverse accessions 2237, 3024 and 2441 were found at different linkage distance ranged from 0.1- 0.2, also including 3022, 2650 and 2616 at 0.075. Finally cluster-8 consisted of maximum number of accessions counted as twenty four. Among these the accessions 3039 and 3056 were recorded at 0.1 and 3037, 3066, 1936, 3017, 3021 and 3040 at 0.075 linkage distance with slight change in their bands. In a similar way, other accessions contained by cluster-8 were 1995, 2855, 3020 and 3035 examined at linkage distance 0.15, determined genetically varied lines when compared their

banding outline with other sorted accessions. Furthermore the accessions 2558, 2188 (C-6) and 3053, 3031, 3063, 2923, 3054, 3045, 3057 and 1998 (C- 8) scored 100% similarity in their banding pattern (Figure 4.17).

Cluster-1 thus grouped 2.8% (USA) of the total accessions and cluster-2 composed of 1.4% (Pakistani) and 2.8% (USA). Cluster-3 showed grouping of 8.6% (Pakistani) with 11.4% (USA) accessions. While cluster-4 identified 5.7% (Pakistani) and 2.8% (USA) lines. Cluster-5 contributed 2.8% (USA) and 1.4% (Pakistani) accessions of the total germplasm used in the study. Cluster-6 and 7 indicated 5.7% (USA) for each and 4.3- 7.1% accessions of (Pakistani) origin. Cluster-8 sorted 28.6% (USA) and 7.1% (Pakistani) lines to evaluate their extent of genetic diversity.

Table 4.13: Sequences of the SSR primers used for molecular analysis of chickpea

S/No.	Name	Sequences Forward/Reverse	Bands	M.Wt (bp)	*t*-value	Coef. Vr.
1	CaSTMS2	ATTTTACTTTACTACTTTTTTCCTTTC AATAAATGGAGTGTAAATTTCATGTA	2	114/110	10.173	82.24**
2	CaSTMS15	CTTGTGAATTCATATTTACTTATAGAT ATCCGTAATTTAAGGTAGGTTAAAATA	1	159	8.547	97.88**
3	CaSTMS21	CTACAGTCTTTTGTTCTTCTAGCTT ATATTTTTTAAGAGGCTTTTGGTAG	1	60	12.689	65.94**
4	TA72	GAAAGATTTAAAAGATTTTCCACGTTA TTAGAAGCATATTGTTGGGATAAGAGT	1	198	39.256	21.31
5	TA130	TCTTTCTTTGCTTCCAATGT GTAAATCCCACGAGAAATCAA	1	219	13.134	63.7**
6	TA194	TTTTTGGCTTATTAGACTGACTT TTGCCATAAAATACAAAATCC	2-3	204/190	4.887	71.19**
7	TA71	CGATTTAACACAAAACACAAA CCTATCCATTGTCATCTCGT	1	202	11.874	70.46**
8	TA22	TCTCCAACCCTTTAGATTGA TCGTGTTTACTGAATGTGGA	1	228	18.262	45.81
9	TA200	TTTCTCCTCTACTATTATGATCACCAG TTGAGAGGGTTAGAACTCATTATGTTT	1	296	19.238	43.49
10	TA46	TTTATTGCAATAAAACTCATTTCTTATC TTCTTTTTGTGTGAAAAAAAAATATAGTA	1	239	16.613	50.36**
11	TA135	TGGTTGGAAATTGATGTTTT GTGGTGTGAGCATAATTCAA	1	192	19.238	43.49
12	TR1	CGTATGATTTTGCCGTCTAT ACCTCAAGTTCTCCGAAGT	1	224	13.134	63.7**
13	TR7	GCATTATTCACCATTTGGAT TGTGATAATTTTCTAAGTGTTTT	1	204	23.125	36.18
14	TR29	GCCCACTGAAAAATAAAAAG ATTTGAACCTCAAGTTCTCG	2	220/270	39.256	21.31
15	TR31	CTTAATCGCACATTTACTCTAAAATCA ATCCATTAAAACACGGTTACCTATAA	1	217	15.906	52.6**

M. Wt-Molecular weight maker: Coef. Vr- Coefficient of variation

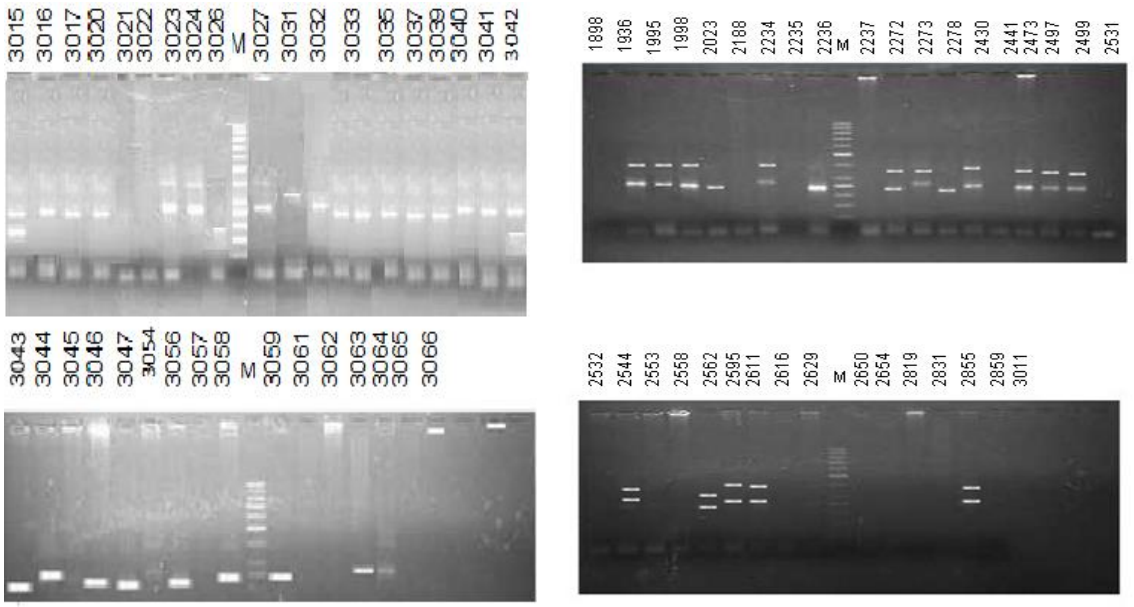


Figure 4.16a: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer CaSTMS2 (114bp) with 50 bp ladder

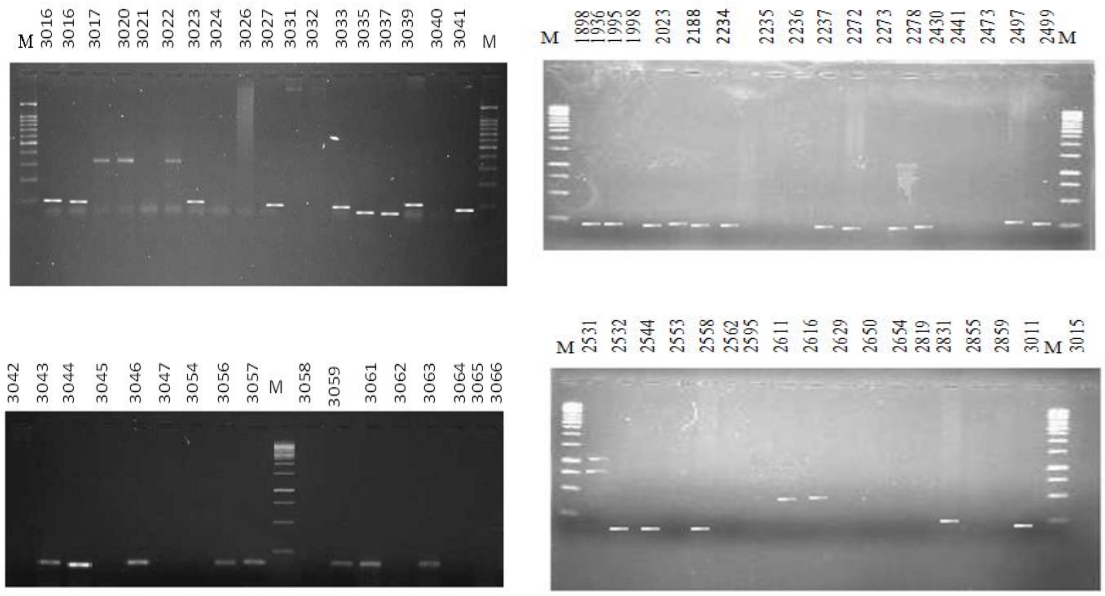


Figure 4.16b: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer CaSTMS15 (159bp) with 1kb ladder

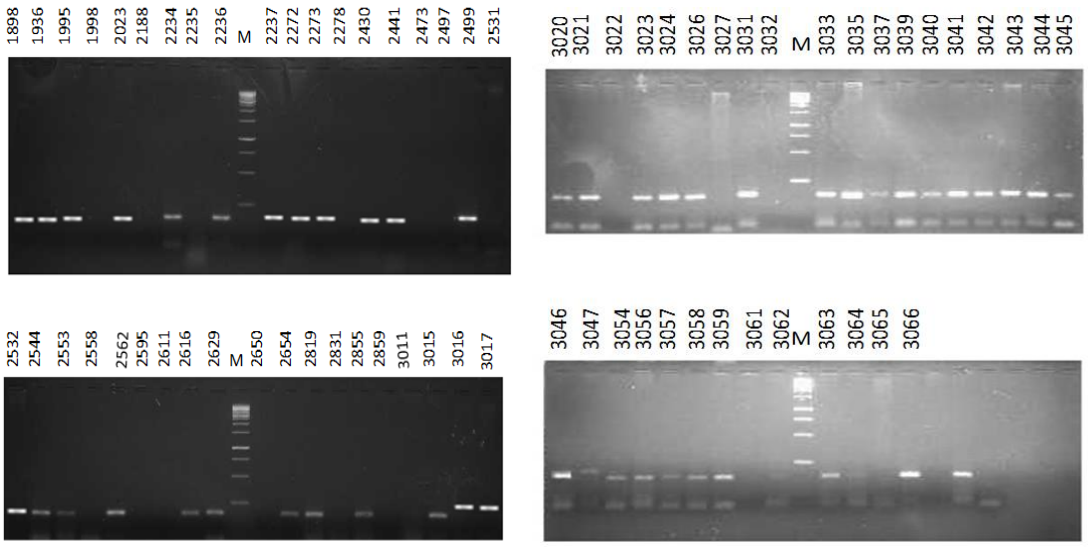


Figure 4.16c: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer CaSTMS21 (60bp) with 1kb ladder

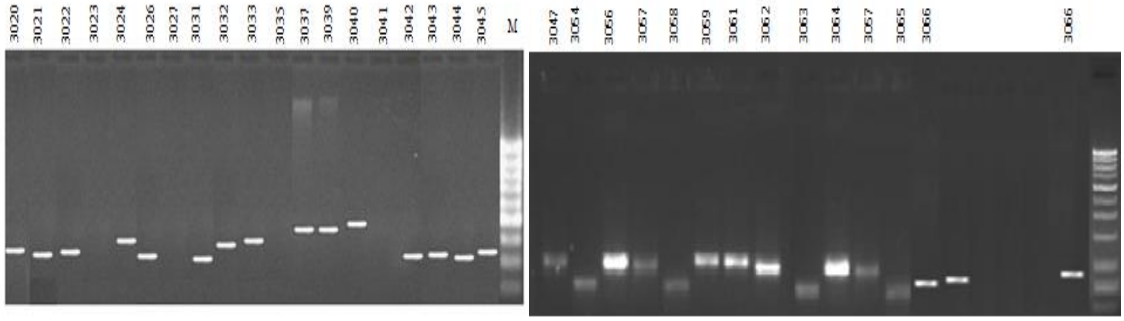


Figure 4.16d: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA22 (228bp) with 1kb ladder

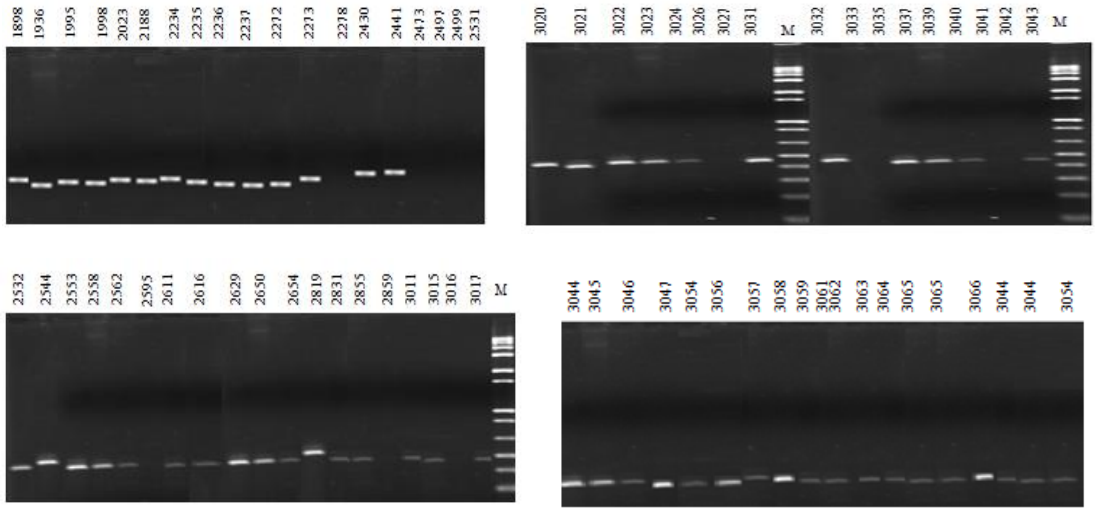


Figure 4.16e: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA46 (239bp) with 100 bp ladder

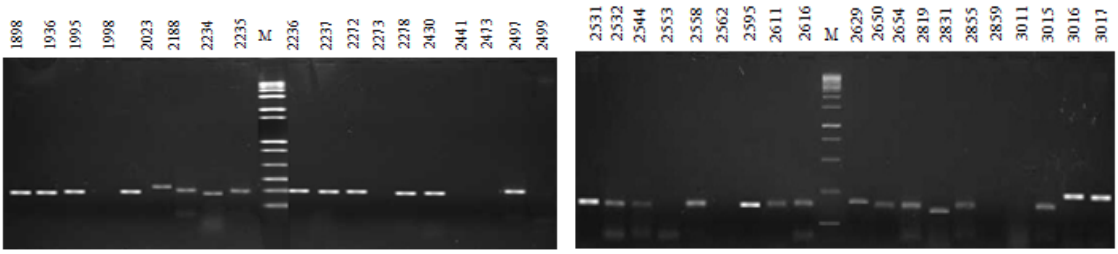


Figure 4.16f: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA135 (192bp) with 100 bp ladder

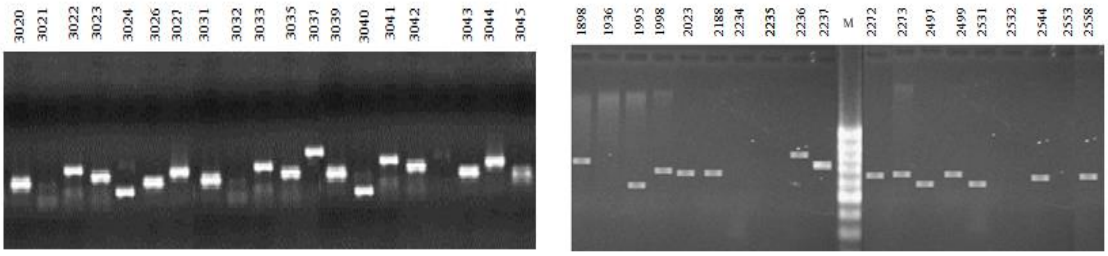


Figure 4.16g: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA135 (192bp) with 100 bp ladder

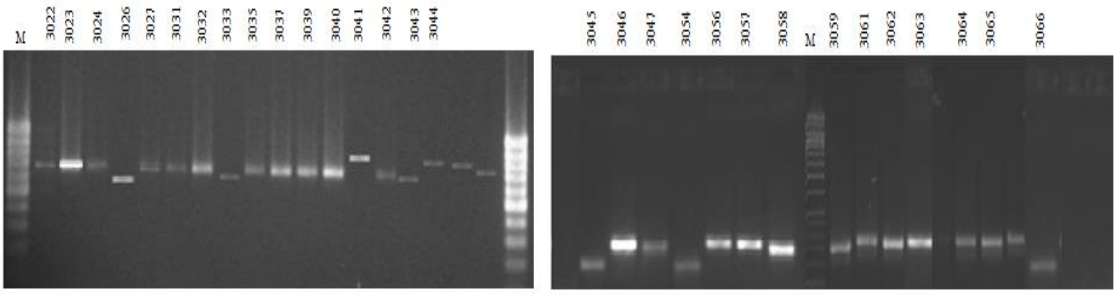


Figure 4.16h: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA200 (296bp) with 50 bp ladder

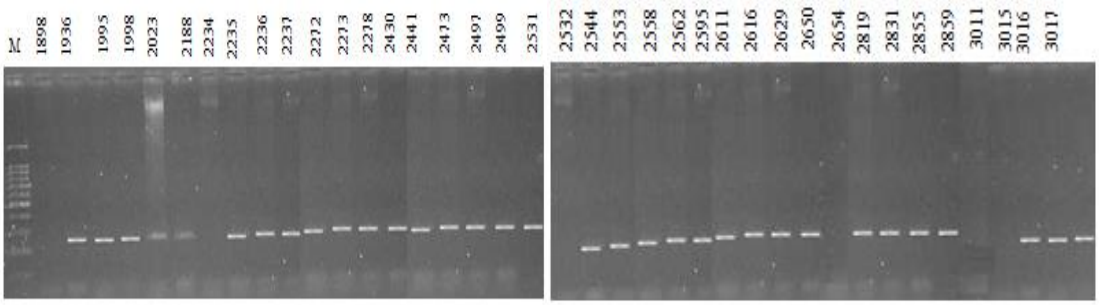


Figure 4.16i: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TR1 (224bp) with 50 bp ladder

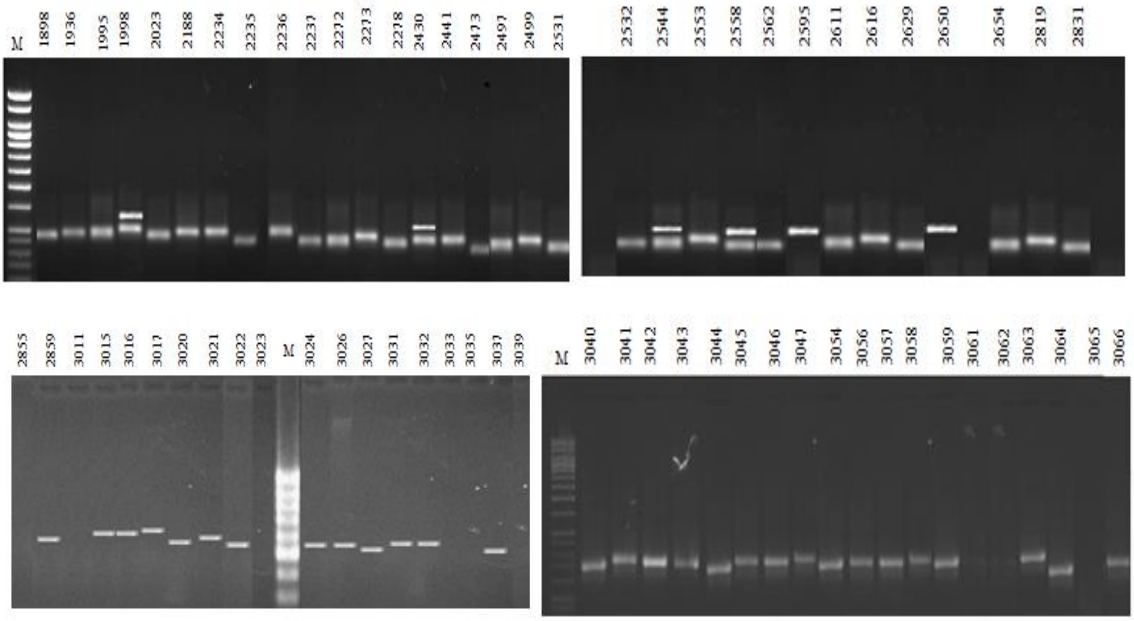


Figure 4.16j: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TR7 (204bp) with 50 bp ladder

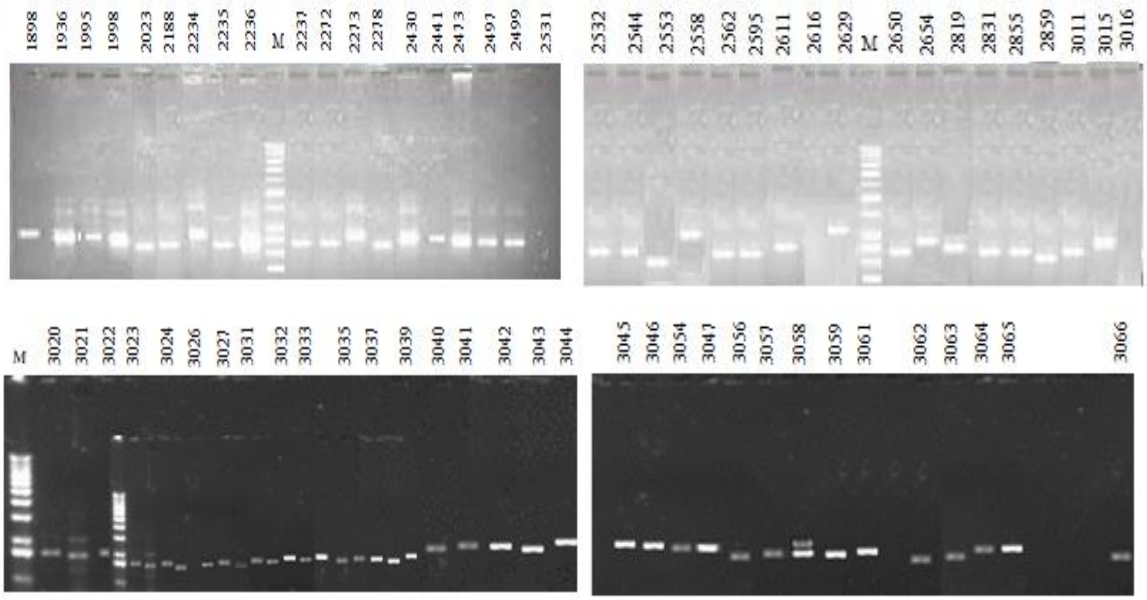


Figure 4.16k: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TR29 (220bp) with 100 bp ladder

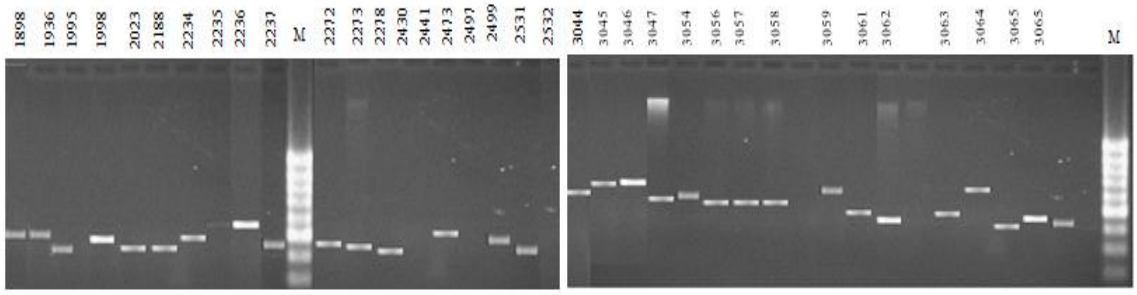


Figure. 4.16L: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TR31 (217bp) with 100 bp ladder

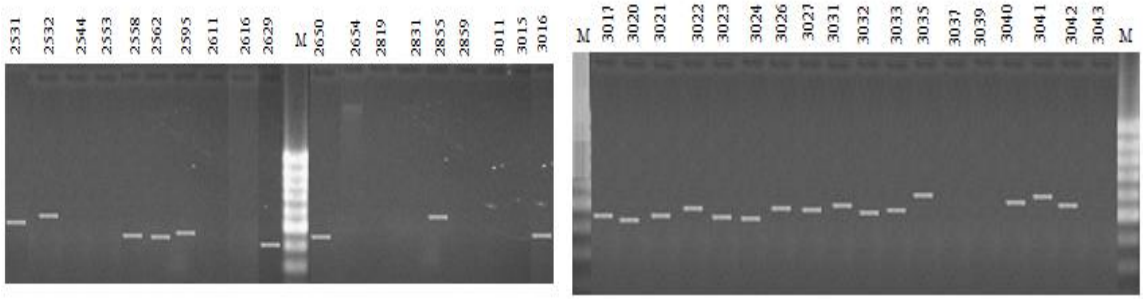


Figure 4.16m: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TR31 (217bp) with 100 bp ladder

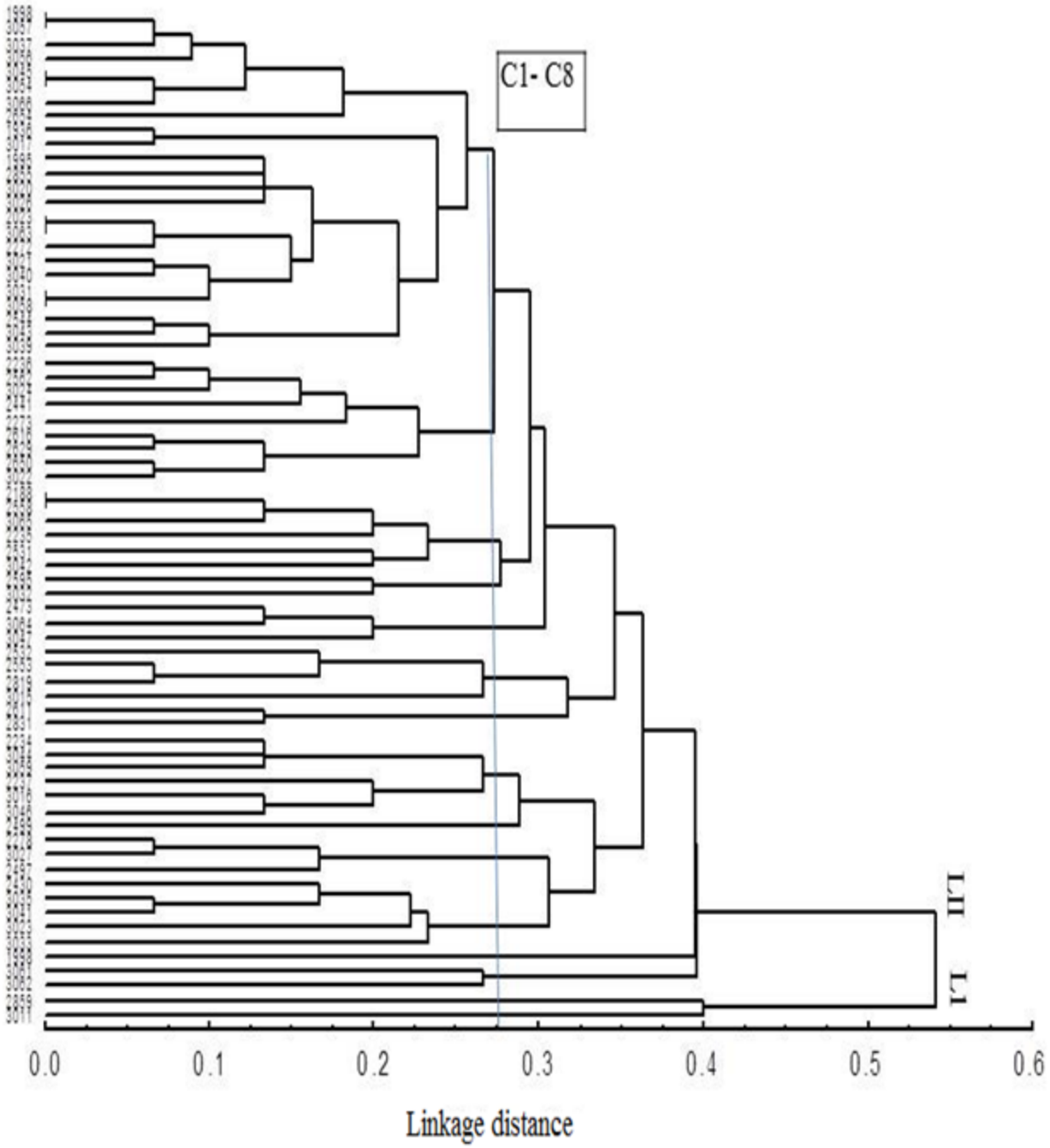


Figure 4.17: Dendrogram of 70 chickpea accessions based on SSR data using UPGMA percent disagreement

4.9 Correlation analysis of RAPD/ SSR markers with yield related traits

In Correlation analysis twenty RAPD and twenty SSR markers were used revealed that only SSR markers TA72 and TA130 were correlated with 100 seed weight and seed size (Figures 4.18- 4.19, appendix 11). To check the linkage distances of the accessions with respect to the linked SSR makers, the banding profile of each utilized makers were scored. The presence of allele (bands) was scored as "1" and absence of allele noted as "0". For estimation of linkage distance the scored data was put in binary data matrix to developed dendrogram based on un-weighted pairs group mean average (UPGMA). Based on genetic distance with respect to genetic disagreement, seventy accessions were grouped into two lineages, L- I and L- II at a linkage distance 0.6. While at a linkage distance 0.4 the lineage-I comprised of cluster-1, based on presence and absence of alleles, enclosed category 2 (Medium) and category 3 (large) seeds with presence of both the alleles for seed weight and size. Cluster-2 of the same lineage included seeds of small size represented by category 1 indicated the allele for seed size only. On the other hand cluster- 3 of lineage- II enclosed medium (category-2) and large (category-3) size accessions with presence of allele for seed weight only. Similarly cluster- 4 of lineage- II, grouped medium, large and small categories of seeds which did not scored any allele for 100 seed weight and seed size (Figure 4.20). The linkage distance of the accessions were retested through Cross validated interpretation of two-way clustering which showed the same pattern of allele distribution (Figure 4.21).

4.9.1 Correlation of yield related traits with SSR markers

The correlation study was carried out among 100 seeds weight, seed size and unique SSR locus 1 and 2. It was found that 100 seeds weight was highly significantly correlated with seeds size; unique locus 1 and 2, at 0.50, 0.64 and 0.69 levels respectively. Furthermore, seed size was also highly significantly correlated with both unique loci at 0.596 and 0.615 levels respectively with $P \geq 0.001$ (Table 4.14, figure 4.21).

Table 4.14: Correlation of 100 seed weight, Seed size and SSR loci in chickpea accessions

Traits	100 seed weight	Seed size
100 seed weight	1.000	0.504**
Seed Size	0.504**	1.000
Unique SSR –locus1	0.644**	0.596**
Unique SSR -locus 2	0.69**	0.615**

**P≥ 0.001 denoted the correlation is significant

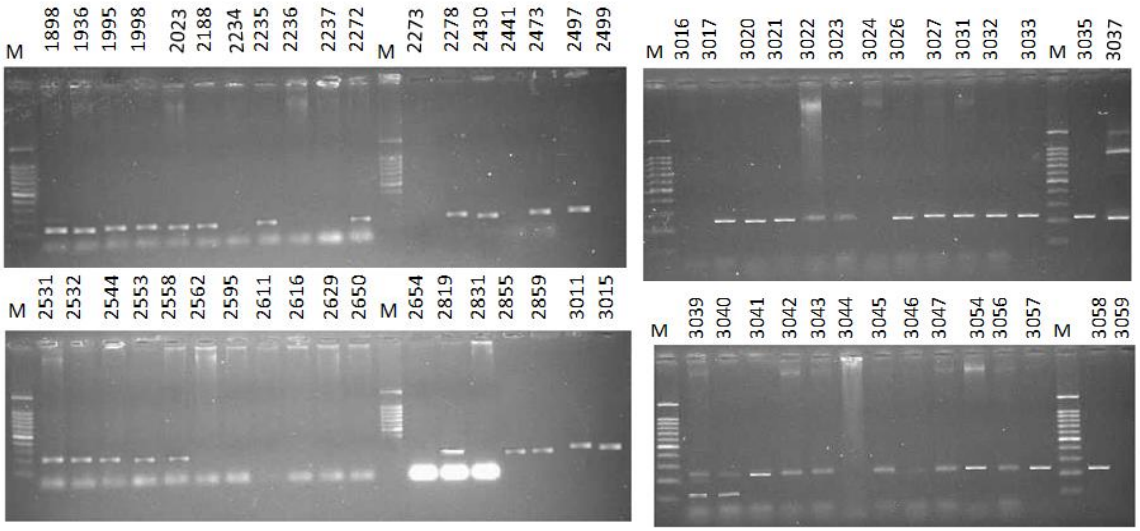


Figure 4.18: SSR-PCR amplification products of chickpea local and exotic accessions using SSR primer TA72 (198bp) with 50 bp ladder

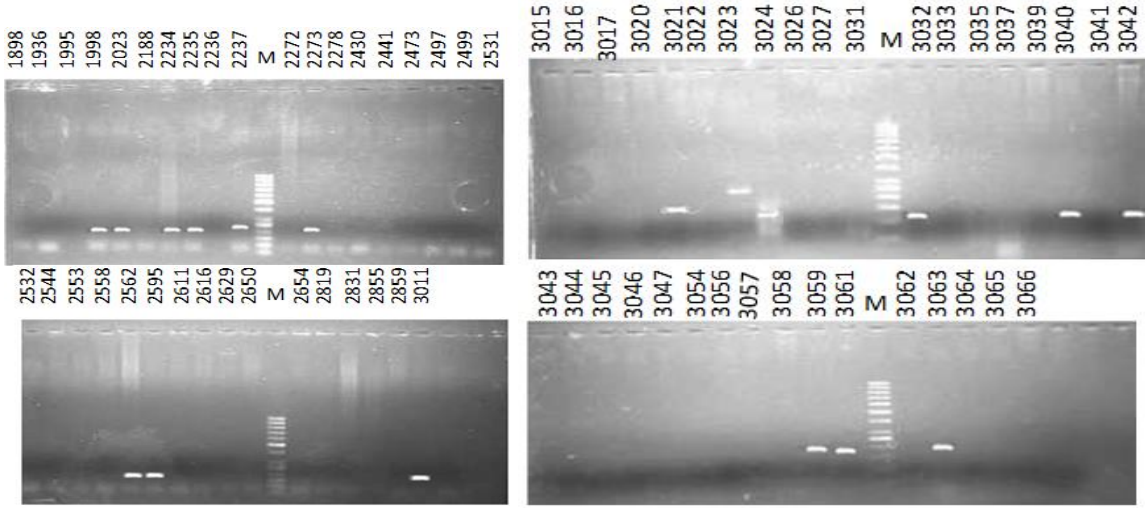


Figure 4.19: SSR-PCR amplification products of chickpea local and exotic accessions using SSR Primer TA130 (219bp) with 100bp ladder

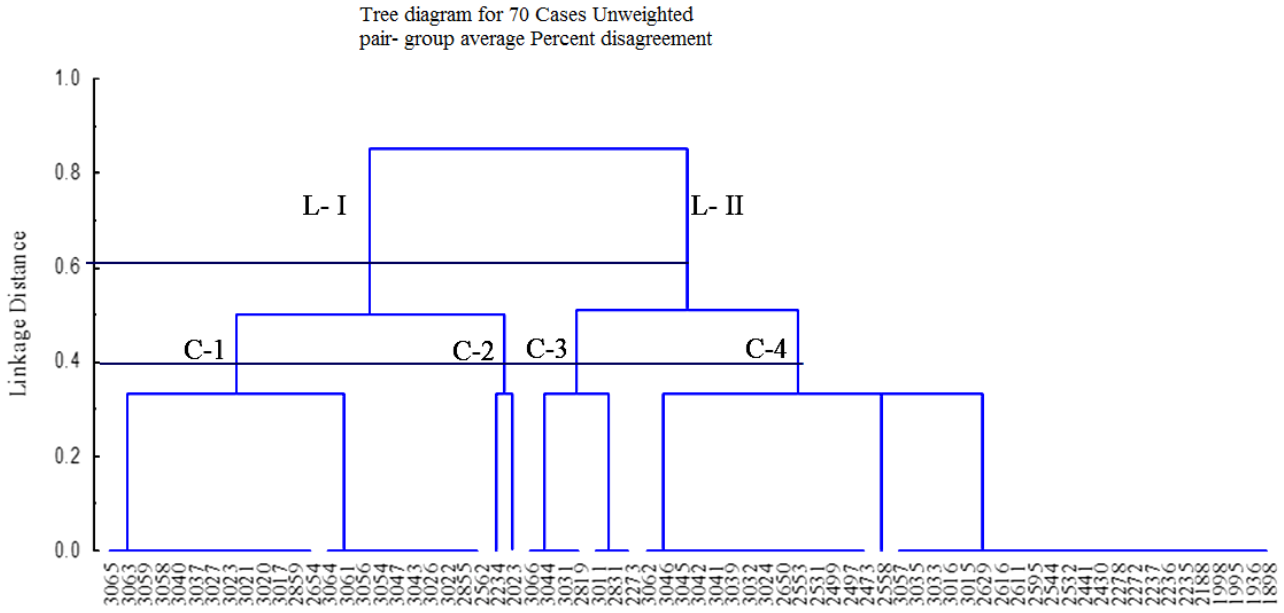


Figure 4.20: Dendrogram of 70 chickpea accessions based on SSR data for 100 seed weight and seed size using UPGMA Percent disagreement

Figure 4.21: Cross validated interpretation of two- way clustering in chickpea 70 accessions based on binary data matrix of SSR makers

4.9.2 RAPD/ SSR markers correlation to wilt resistance gene

To further evaluate and identified wilt resistance lines among chickpea germplasm, five RAPD and fifteen SSR markers were investigated to assess their correlation or association with *Fusarium* wilt resistance gene. These primers were selected from previous literature (Agrawal *et al*., 2006; Iruela *et al*., 2007; and Datta and Lal, 2011). However in the present study the SSR marker TA194 has only shown significant relation with the presence of allele for resistance (Table 4.15, appendix 10), therefore, it has been selected for further analysis. The dendrogram constructed on the basis of coefficient of similarity using UPGMA divided the total germplasm into two lineages and four clusters resulted in splitting of 70 accessions into two groups. The first group displayed 78% accessions resistant to wilt disease, while the remaining 21% grouped as susceptible (Figure 4. 22). The correlation probability of TA194 marker was 85% (Table 4.16), and this association of the marker was reconfirmed by ROC curve (Figure 4. 23). Thus the coefficient of correlation of marker TA194 with disease resistant gene (*FOC* locus), Factor 1 was highly significant at P≥ 0.01 (Table 4.15). The PCR amplification using TA194 however; for certain accessions have shown multiple bands (Figure 4. 22).

Table 4.15: Coefficients of correlation between resistance and allele based on SSR in chickpea

	Estimate	Std. Error	z value	Pr (>|z|)	
(Intercept)	-1.8718	0.7596	-2.464	0.0137	*
factor(allele)1	3.6425	0.8504	4.283	1.84e-05	***

Table 4.16: Association of level of probability of resistance with presence of allele based on SSR in chickpea

A	-1.8718		
b (allele1)	3.6425		
probability of resistance when allele is present	p=e^(a+b)/1+e^(a+b)	0.85468	0.854
probability of resistance when allele is absent	p=e^a/1+e^a	0.1332	0.1333

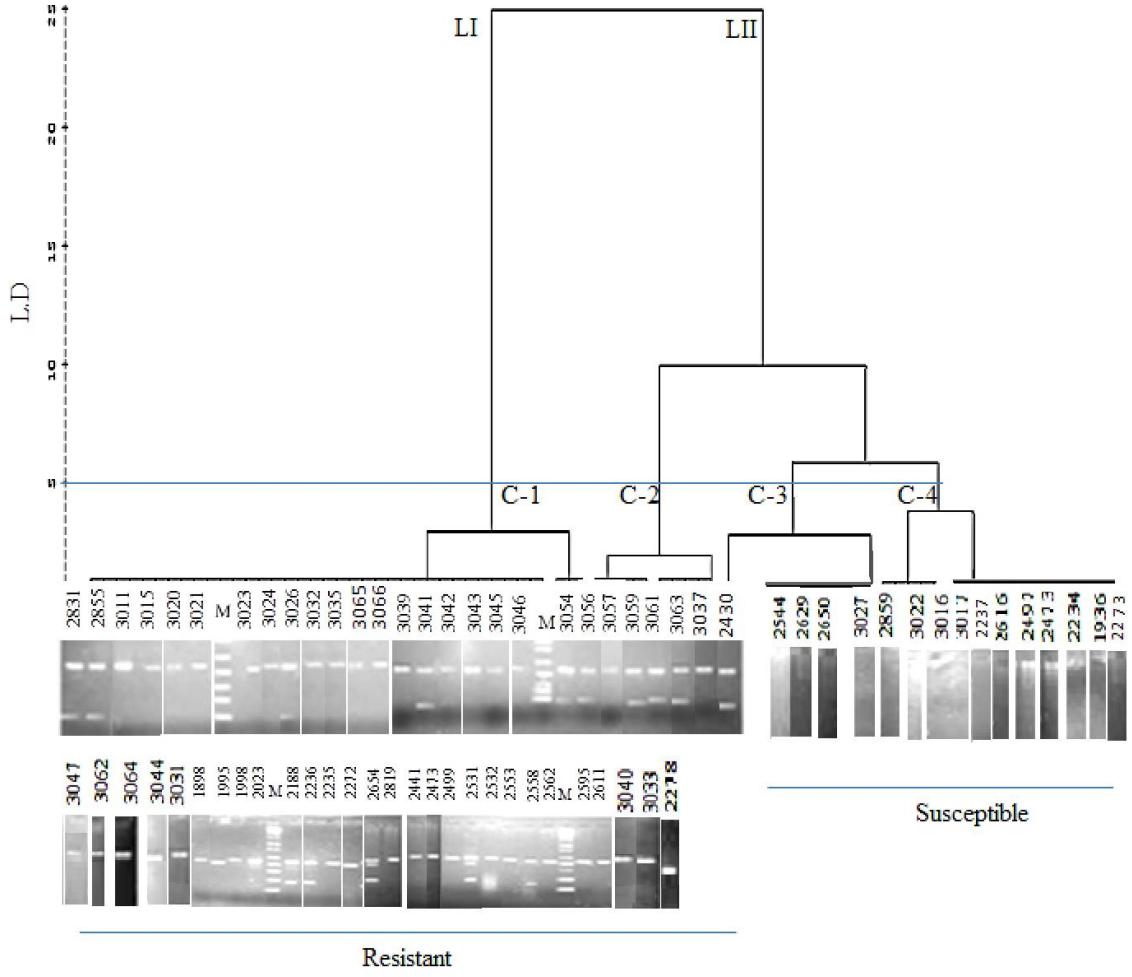


Figure 4. 22: Comparative representation of field screening and PCR data for delimitation of resistant and susceptible accessions of chickpea germplasm.

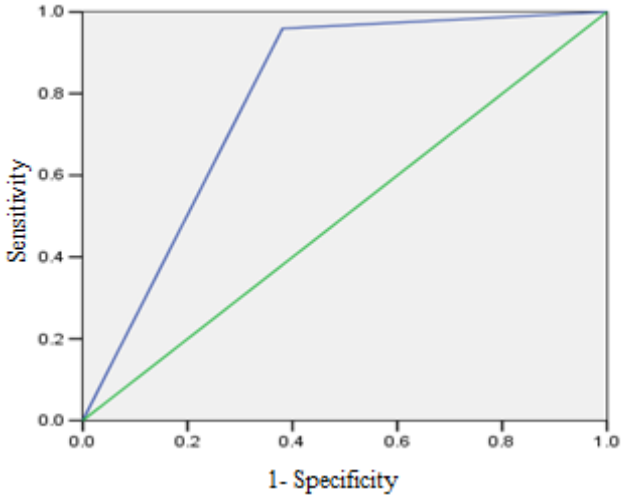


Figure 4. 23: Receiver operating characteristic (ROC) curve to show range of resistivity against *Fusarium* wilt in the presence of resistant gene in chickpea accessions.

DISCUSSION

The diversity in global collection of the mandate crop *Cicer* is represent by its developed reference set which is globally available and its core and mini-core collections for crop improvement (Gaur *et al.,* 2010). However several challenges are there for *Cicer* as a crop. There are several pests and diseases of chickpea (such as *Aschochyta* blight and *Fusarium* wilt). Therefore, the development of accession level data and subsequent comparison of these data across collections would greatly facilitate the identification of unique accessions. Further work is needed on characterizing and evaluating the collections and on making the information openly available. Through the generation of accession-level data and by improving the accessibility of information on accessions globally, unique genetic resources may be identified and prioritized for support. Only followed such efforts, as well as the formation of stronger collaborative relationships with user communities, is there likely to be a significant increase in the use of collections by plant breeders and others.

5.1 Morphological characterization

Morphological characterization is a first step in classification and description of crops and the assessment of variability in phenotypic traits are the foremost interest to consumers (Farshadfar and Farshadfar, 2008). The losses in morphologically valuable characteristics at a higher rate in any plant population greatly affect the production of crop plants (Trethowan and Kazi, 2008). Therefore, the survival of plant populations requires a high level of genetic diversity. This has also been reported by several authors in providing genetic barriers against disease management and improvement of plant species (Hughes *et al*., 2004; Hajjar *et al*., 2008). The coefficient of variation in agronomic traits has widely been used to determine variations available in populations. In the present study among qualitative traits a maximum range of coefficient of variation (%) has been shown by seed color (79.52 %) followed by flower color (77.41%) agreed with the observations of Khan *et al.* (2011). In addition, 44.8% and 56.8% average

coefficient of variations were scored for qualitative and quantitative traits respectively. This level of variation in the study indicates the strength and potential of the collected germplasm.

Generally, these results indicated there is variability within chickpea. Kumar *et al*. (1981) also reported a high coefficient of variation for quantitative traits related to yield. Thus, there is genetic variation among chickpea genotypes and selection could be effective for such traits (Khorgade *et al.* 1985; Pandey and Tiwari, 1983; Arora, 1991).

On the other hand, previous research reports showing that association between traits varied with location and years (Van der Maesen, 1984; Abebe, 1985). From the description of Abebe (1985), yield and yield component association showed differences in different seasons, environments and locations, which is signified by the variation observed between grain yield and 100 seed weight, plant height and primary branches, seeds per pod and number of pods per plant. Van der Measen (1972) also suggested that seeds/pod may not be always associated strongly and positively with yield of chickpea. Generally, as depicted from such results, the correlation coefficients may reveal differences in magnitude over location and seasons that might be attributable to the environmental conditions prevailing at different locations and seasons. This implies the need for determining the association among the important traits over broad arrays of environment and seasons for identifying consistent association among traits that could be used for conducting effective breeding programs.

Significant correlation in yield contributing traits was observed which is helpful for the establishment of traits improvement. In correlation studies 100 seed weight was found highly significantly positively correlated with grain yield in 2008-2011, while, positively significantly correlated with total biomass in 2010. Similar findings were also reported by Phadnis *et al*., 1970; Singh *et al*., 1983; Malik *et al*., 1987; Ghafoor *et al*., 1993; Sarvaliya and Goyal, 1994 and Aziz *et al*., 1995. Similarly grain yield has positive significant correlation with harvest index in 2008- 2009 and 2011, while this relation was highly significant with the same trait during 2010. These results are in close agreement

with Cakmakei *et al*., 2003 and Ghafoor *et al*., 2000. Total biomass however, observed negative correlation with harvest index during a year 2009, 2010 and 2011.

According to the observations of Dangi *et al*., 2004, the composition of genotypes is greatly affected comparatively by its high level of environmental error (EE). Genetically well-defined and diverse cultivars of specific areas always showed significant variation in their biological efficacy and chemical composition of the plant products (Acharya *et al*., 2010).

Traditionally phenotypic observations are usually used for characterization of plant varieties (Castro *et al*., 2010). This study identified 11 accessions based on their performance and stability in valuable quantitative traits during three consecutive years *i.e.*, 2008-11. The accessions were 3022, 3040, 3059, 3063 (For total biomass), 2819, 3039, 3056 (For 100 seed weight and grain yield), 1898 (For Harvest index), 3037 (For 100seed weight and total biomass), 3043 and 3054 (For 100 seed weight, grain yield and total biomass). On the basis of which the promising lines can be selected for chickpea future breeding programs. Among these 11 accessions; lines 3054 and 3043 of USA origin could be used directly as cultivars because of their best performance agronomically. These two selected lines will be grown in field trials for screening against diseases and multiplication for ultimate use of farmers. Results showed that the majority of these promising lines are of USA origin apart from a single accession 1898 which is of a Pakistani origin. Such types of morphological markers have also been considered by Khan *et al*., 2008; Saleem *et al*., 2008; Durga *et al*., 2007; Hakim *et al*., 2006 and Ghafoor *et al*., 2004 to evaluate the variability among chickpea lines. Crop stability exhibits the progress in selection of best genotypes and minimum interaction with environment (G x E). Varieties with low G x E interaction usually have a high stability in yield and production. Thus low level of interaction indicates less environmental influence on the performance of accessions and the yield is largely affected by the genetic composition of the accessions (Tai, 1971). It is an important research study to observe the performance stability among various genotypes to select more superior and disease free

chickpea lines for future breeding programs (Bakhsh *et al*., 2011). The Significant correlation in yield contributing traits is always helpful for the establishment of trait improvement. In correlation studies 100 seed weight was found positively highly significantly correlated with grain yield (Ahmad *et al*., 2012). Seed weight a valuable quantitative trait was also proposed as an accurate measure of chickpea seed size (Upadhyaya *et al*., 2006). Therefore, to produce seed of an ideal size, and to meet a specific market demand through targeted breeding, knowledge about seed size inheritance is required. A large seed size variation exists however in both desi and kabuli types of chickpea (Hossain, 2010). In the present investigation 100 seed weight was found to be a stable trait and positive significantly correlated with seed size, also reported by Bicer, 2009. Chickpea crop is suffering by a number of prodigious disorders including multiple disease stress and other environmental stresses which directly affecting upon the yield (Jenkins, 2011). It is evident from the study that medium and large size chickpea accessions have conquered maximum 100 seed weight, range from 30- 57gm. The majority of accessions of USA origin were observed with maximum 100 seed weight and medium to large size seeds including one of the accession 2562 of Pakistani origin also with large size seeds. Thus the accessions 3037, 3040, 3065, 3027, 3063, 3059, 3058, 3021, 3023, 3020 and 2654 (USA) attained seeds of medium size ranged from 4.2-7.2mm with mean value of 100 seed weight 56.66, 44.27, 39.37, 37.65, 37.11, 35.51, 32.53, 31.77, 31.72, 31.12 and 31.15gm respectively. While the accessions 3056, 3054, 3043, 3026, 3047 and 3064 also of USA origin were found in the category of large seed size range from 8-9.9mm. The maximum mean values of 100 seed weight for which have been calculated as 57.18, 53.16, 46.19, 37.63, 30.92 and 30.71gm respectively. These results revealed that the use of larger and medium size seeds may enhance the yield. Secondly the higher yielding and best performance showing chickpea accessions, particularly the exotic germplasm of USA origin, are key resources to improve chickpea cultivation in Pakistan. Similar findings were obtained by Stougaard and Xue, 2005 and Royo *et al.*, 2006, in wheat by estimating 18% increase in yield while using seeds of larger size and 16% decrease with the use of small size seeds. Among the total evaluated

germplasm a high (44%) frequency was recorded for medium size chickpea lines followed by small and larger type of seeds. However, 100% range of cumulative frequency was observed in case of large size seeds indicating an excellent future trend in chickpea yield enhancement.

5.1.1 Screening of chickpea for wilt resistance

There are biotic and abiotic stresses which contribute to low yield of chickpea production and for their control field diagnosis of chickpea diseases were made by different scientist (Nene *et al.,* 1991). Chickpea wilt has a major threat when weather conditions are conducive in Pakistan. The disease is favoured by drought and high soil temperature ranging from 25-35°C. It can cause 55-95% mortality of chickpea seedlings (Gurha and Dubey 1982). Screening studies of chickpea for wilt resistance were made during the years 2012 and 2013. A set of 70 germplasm accessions were evaluated in the greenhouse as well as in the field. By incorporation of identified resistance genes numerous resistant varieties have been released. In field screening the genotypic response to disease revealed that 20 accessions were highly resistant, 20 were resistant, 9 moderately resistant or tolerant and 21were susceptible at the seedling stage. Whereas, 14 accessions were highly resistant, 17 resistant, 9 tolerant and 30 was susceptible at the reproductive stage. Classification of disease reactions were made according to the percentage of dead plants at physiological maturity of each genotype (Nene and Haware, 1980). Similar studies have also been reported by others (Chaudhry *et al.,* 2006; 2007; Infantino *et al*., 2006; Reddy *et aI.,* 1990; Govil and Rana, 1984).

In the greenhouse the inoculum was mixed thouroughly in the entire soil surface with profuse growth of the pathogen, therefore there was not any chance of the seedlings to escap from the disease. This technique is much faster than those used by other workers (Gurha and Dubey, 1982). This procedure was also used by Sugha *et al.* (1991) who tested 210 lines/ varieties of chickpea for the resistance and obtained consistent, quick and repr`oducible results. Other techniques are time consuming, requiring much inoculum and are less effective. These do not give consistent and reproducible results as

compared to the sorghum grains infested with pathogen and mixed in soil. Similarly, in different countries, desi and kabuli chickpea cultivars having resistance against *Fusarium* wilt were identified (Elfatih *et al*., 2002; Jimenez-Diaz *et al*., 1991; Buddenhagen *et al*., 1988; Murumkor and Chavan, 1985; Kumar *et al*., 1985; Halila *et al*., 1984; Haware and Nene, 1980).

Chickpea genotypes have been demonstrated to differ in time of wilting. It was observed that out of 70 accessions 18 were highly resistant, 32 resistant, 14 moderately resistant or tolerant and 6 were susceptible at a seedling stage under greenhouse conditions. Wherease, 15 accessions were found highly resistant, 26 resistant, 12 moderately resistant or tolerant and 17 susceptible at reproductive to pods maturity stage. The wilt incidence was calculated 30% in field screening of germplasm at seedling stage and this condition increased 42.85% at reproductive to pod maturity stage. Contrary to this greenhouse conditions, it was observed much reduced up to 8.57% at seedling stage and 24.28% at reproductive stage. This increase in susceptibility to wilt disease was observed that may be due to slow wilting resistance of certain chickpea accessions require long time to wilting. The *t*-test however, indicated that chickpea both from indigenous and exotic origin showed significant variation at alpha ≤ 0.050 at seedling and reproductive stage that has already been reported by Ansar *et al.* (2010). The results of the present study coincide with the previous work done by others for resistance. Zote *et al.* (1983) reported that four lines having less than 10% and other six has less than 29% disease from 42 lines of *C. arietinum* in a wilt infested plot. Govil and Rana (1984) screened 239 cultivars of Indiana Iranian germplasm and found P-597, P-621, P-3649, P-4128 and P-4245 resistance from Indian cultivars. Zote *et al.* (1986) tested 15 lines for three successive years and reported five chickpea lines having less than 10 percent wilt incidence. Chickpea screening against *Fusarium* wilt revealed that the incidence and the severity of the disease were high in the field. One of the reasons might be that crop often has the chances of disease escape, as the wilt disease is temperature dependent and the level of inoculum may vary at different places. Our results indicate that occurrence of resistance in chickpea germplasm to *Fusarium* wilt is not uncommon. Similar studies

have been made in different countries (Yu and Su, 1997; Iqbal *et al*., 1993; Ahmad and Sharma, 1990; Kaushal and Singh, 1990; Reddy *et al*., 1990; Ahmad *et al*., 1990; Zote *et al*., 1983; Pathak *et al*., 1982, Shah, *et al*., 2009). Babu and Ravikumar (2009) found least pollen tube growth inhibition in resistant genotypes whereas it was more in susceptible genotypes due to fusaric acid. Resistance in chickpea wilt is either due to monogenes or oligogenes. Late wilting is due to individual genes of oligogenic resistance mechanisms which delay the onset of disease symptoms. Slow development of disease occurs after pathogen reaction took place (Sharma and Muehlbauer, 2007). For complete resistance two genes are involved in *Fusarium* wilt, the one of which is in homozygous recessive form, whereas, the other is homozygous or heterozygous in dominant form. When both the loci are dominant late wilting took place, whereas, early wilting occurs by a homozygous recessive gene (Gumber *et al*., 2008). The economical and the most ideal way of managing chickpea wilt is the use of resistant cultivars, which are not common in the existing chickpea germplasm. The present study revealed some useful accessions in the chickpea germplasm, which were resistant against local isolates of the fungus, so these could be exploited for breeding against chickpea wilt resistance.

5.2 Genetic diversity based on SDS-PAGE

Seed storage proteins are largely free from environmental influences, therefore, considered as a reliable tool for estimation of genetic diversity in crop plants (Nisar *et al*., 2007). SDS-PAGE has been successfully used by many researchers *e.g.,* Ghafoor *et al*., 2003; Iqbal *et al*., 2005; Nisar *et al*., 2007; Netra and Prasad, 2007; Hameed *et al.*, 2009; Nisar *et al*., 2011) to resolve the evolutionary and taxonomic problems for delimitation of taxa. However, few studies are not in favour of the above statement because electrophoretic patterns of proteins are often similar among cultivars (Ladizinsky and Alder, 1975; Raymond *et al*., 1991; Ahmad and Slinkard, 1992 and De veries, 1996).

In the present study the SDS-PAGE analysis showed 50% genetic diversity among 70 chickpea accessions of USA and Pakistani origin. This considerable variation; as not based on their geographic distribution and moreover, these lines were not separated

on the basis of their performance. The dendrogram developed based on (UPGMA) percent disagreement reported the accession 3027 with a promising line 3045 of USA as unresolved. Cluster-1 comprised 5 USA and 4 Pakistani accessions constituting 13.2%, cluster-2 grouped 33 USA and 13 Pakistani accessions contributed 67.6% and cluster-3 consisted of 6 USA and 7 Pakistani accessions constituting 19.1% of the total germplasm. The promising lines, 2819 and 1898 occupied cluster-3 also indicated close relationship on the basis of their banding profile. On the other hand 3039, 3037 and 3063 of USA were found to be closely related with 13 Pakistani accessions of cluster-2. The accessions 2629, 2595, 3042, 3037, 3015, 2859, 3047, 3032, 3040, 3033, 3063, 3062, 3061, 3054, 2855, 2831 and 2819 were of USA origin however, showed 100% similarity in their protein data with the accessions 2562, 2497, 2473 of Pakistani origin and both of them forming 28% of the total germplasm. Similarly Pakistani accession 2273 was observed at the same linkage distance 0.275 with 3044, 3011 and 3017 of USA which showed greater variation from other accessions selected for this study.

5.3 Molecular characterization

Besides morphological and biochemical characterization of germplasm for evaluation of genetic diversity among chickpea indigenous and exotic accessions, the molecular marker-based genetic diversity was examined which is necessary in breeding for marker assisted selection and genetic mapping (Lapitan *et al*., 2007)**.** The two marker system of RAPD and SSR have been successfully used by very few researchers either in combination or singly in comparative studies on legumes (Ravi *et al*., 2003; Souframanien and Gopalakrishna, 2004; Gillaspie *et al*., 2005; Dikshit *et al*., 2007; Vural and Akein, 2010; Mahmood *et al.,* 2011). The PCR based marker system can be used for diversity analysis in legumes to select parents for breeding purposes and for generation of mapping populations (Datta and Lal, 2011).

5.3.1 Genetic diversity using RAPD markers

Traits determined by RAPD technique are highly polymorphic and useful in studies on chickpea concerning its genetic diversity, phylogeny and evolutionary biology (Iruela *et al*., 2002). Among twenty RAPD markers, only five were found to be polymorphic and showed 37% genetic diversity in total germplasm. These markers revealed a considerable amount of variation in the sampled genome. These results suggested the presence of useful genetic diversity both in indigenous and exotic breeding line resources. There is a need for further evaluation of the molecular genetic diversity through the application of additional markers to improve chickpea genome. Such efforts will address the current concerns on the narrowness of the genetic base of chickpeas. However, studies of the estimated molecular genetic diversity still offer a useful guide for chickpea breeding; as such studies are more informative than selection and traditional pedigree analysis. In addition among RAPD markers UBC181 and UBC733b have shown 89.80% and 77.43% allele polymorphism respectively. The dendrogram based on RAPD markers displayed the genetic relationship among chickpea accessions, is accorded with the previous investigations on chickpea lines (Ahmad *et al*., 1992; Tayyar and Waines, 1996; Iruela *et al*., 2002).

The dendrogram divided total germplasm into seventeen clusters, in which cluster-12a grouped 34.2% accessions, scored highly polymorphic bands at different linkage distances. On the other hand, cluster-4 having accessions, 2558 and 2553of Pakistani origin, with 22% dissimilarity in their banding profile, which was found as 25% in 2497. It was evident from the study that the results obtained from morphological markers were quite comparable with those of biochemical and molecular markers because in the present study cluster-12a scored the promising lines 3059 and 3043 at a linkage distance 0.14, 3039, 3063 at 0.20 and 3056 at 0.13 of USA were closely allied indicating that there is a strong link between morphological and molecular markers. These results proved RAPD markers to be good indicators of morphological divergence (Talebi *et al*., 2008). Similarly, 3024 (cluster-9), 3065 (cluster-14), 3054, and 3037 (cluster-5) of USA resulted in 29% diversity, determined a high degree of polymorphism

in their banding pattern. Generally, this result indicated that RAPD markers could be used for discriminating chickpea population for analysis of chickpea diversity.

5.3.2 Genetic diversity using SSR markers

The analysis performed using SSR markers for detection of genetic diversity among indigenous and exotic lines could differentiate the accessions on the basis of unique or rare alleles have also been reported by Varshney *et al*., 2007 and Joshi *et al*., 2010. In fifteen SSR markers out of twenty have displayed polymorphic informations about the germplasm, while the remaining five were not considered for further analysis due to their poor amplification and repeatability. The level of genetic diversity among chickpea cultivars estimated as 55% using SSR markers, in which the size of certain amplicons was highly variable and accorded with the observations of Datta and Lal, 2011. The primers CaSTMS2, CaSTMS21, TR7, TR29 and TA194 have shown multiple bands, reported earlier by Holton *et al*., 2002 in their studies; where SSR markers amplified more than one locus. One of the reasons for the appearance of multiple bands is the presence of cryptic sites of the primer binding sites (Winter *et al*., 1999). Among the SSR markers CaSTMS15, CaSTMS2, TA194 and TA71 have shown higher (%) of coefficient of variation calculated as 97.88%, 82.24%, 71.19% and 70.46% respectively. Thus the significant coefficient of variation found among the accessions based on loci presence and absence determined stability by measuring genetic diversity in chickpea accessions.

Cluster analysis showed that cluster-8 scored a maximum number of genotypes and promising lines of USA origin. Among these lines 3039 and 3056 were found at a linkage distance 0.075 were also grouped together in case of RAPD analysis. In the present investigation, SSR markers failed to amplify the genomic DNA of certain accessions and they did not present the specified band at all; may be due to mutation in primer binding site or absence of the locus (Datta and Lal, 2011). Simple sequence repeat or SSR is considered a more powerful technique having the advantage of co-dominant markers for efficient detection in both homozygotes and heterozygotes (Datta and Lal,

2011). In this study, SSR markers detected high genetic diversity (55%) among chickpea accessions as compared to RAPD and SDS-PAGE which was calculated as 37% and 50% respectively.

The characterization of chickpea accessions using SSR markers provided a useful guide for selecting specific germplasm with distinct genetic backgrounds in efforts to diversify chickpea breeding programs. The current study showed that microsatellite markers are efficient for measuring the genetic diversity and relatedness for identifying high yielding chickpea accessions. This is because of the narrowness of the genetic diversity in the germplasm was associated with the recent and potentially future declines in *C. arietinum* L. production and its quality, serving as a timely warning to increase the speed of the efforts to widen the genetic base of the germplasm resource by mobilizing new genetic variations from the gene pool.

5.4 Marker trait association/ correlation

5.4.1 Marker assisted selection for seed size and seed weight

In marker trait correlation analysis of 20 RAPD and 20 SSR markers, only SSR markers TA72 and TA130 have shown association with 100 seed weight and seed size respectively. The dendrogram however constructed based on UPGMA percent disagreement characterized by the presence and absence of specified alleles using TA72 and TA130 resulted in a grouping of 70 accessions into four clusters. This grouping was based either on the presence of loci for both the selected traits (100 seed weight and seed size) or for a single trait only (100 seed weight or seed size). In addition, cluster-1 related to the group of medium and large size seeds and the presence of both loci for allele 1 and 2, indicate that the accessions with high (%) of seed weight have also shown larger seed size irrespective of desi or kabuli type. Similar markers were also reported by Hossain, 2010, who determined that the inheritance of 100 seed weight was not influenced by any environmental factor and the small sized seed character is due to maternal effect which prohibited the full expression of larger seed size. These results were on accord with the findings of Rastogi, 1979; Malhotra *et al.*, 1997 and Kumar and

Singh, 1995. While Niknezad *et al*., 1971 observed larger seed size dominant over small size.

5.4.2 Marker assisted selection for *Fusarium oxysporum* wilt resistance

The MAS enhance sources of distinction and made the complex traits selection easier which is otherwise time consuming process when evaluated phenotypically. The procedure of MAS for disease resistance which is typically a quantitative trait can be more efficiently developed (Calonnec *et al.,* 2012) and stability among various genotypes to select superior and disease free chickpea lines is the key criterion for future breeding programs (Bakhsh *et al*., 2011). A high level of resistance in chickpea genotypes against *Fusarium* wilt disease has been studied (Ahmad *et al*., 1990; Reddy *et al*., 1990; Ahmad and Sharma, 1990; Iqbal *et al*., 1993; Yu and Su, 1997; Iftikhar *et al*., 1997). But identification and evaluation of chickpea wilt resistant lines against *Fusarium oxysporum* f. sp. *Ciceri (FOC)* aiming at combined field screening linked with gene using PCR based markers is a new avenue in chickpea breeding in Pakistan.

For more efficient procedure to identify chickpea resistant lines in the available germplasm against *Fusarium* wilt disease the molecular markers can be used for chickpea screening to facilitate gene pyramiding and molecular breeding (Soregoan and Ravikumar, 2010). The previous workers (Sharma and Muehlbauer, 2007; Gowda *et al*., 2009; Soregoan and Ravikumar, 2010) identified the genetic linkage of resistant genes using different RAPD and SSR markers for various *FOC* races (*FOC*1, 2, 3, 4 and 5) in inbred chickpea lines developed from resistant and susceptible parental combinations. While, in this study it has been observed that among the molecular markers (20 RAPD and 20SSR markers) *i.e.,* TA194 at a molecular weight 204bp showed a correlation with chickpea germplasm that was not reported earlier. Thus it was suggested that this SSR primer that successfully separated resistant (1) and susceptible lines with significant association/correlation with allele for resistance should be practically utilized for target chickpea breeding resistant to wilt. The results based on the dendrogram, were quite comparable with field observations. The dendrogram separated 78% accessions of the

total germplasm which maintained their resistance response. While the remaining 21% accessions which did not show the presence of any allele for resistivity, have also been examined susceptible to the disease in field trial. Furthermore, the linkage probability of TA194 marker was 85%. This significant linkage of primer with resistivity against wilt disease was reconfirmed by receiver operating characteristic (ROC) curve analysis which is recently developed in numerous agricultural applications for evaluation of performance of diagnostic experiments in the form of graphical representation (Yuen, 2006; Dewdney *et al*., 2007; Wray et al., 2010; Wang *et al*., 2012). A Similar analysis has also been performed by Calonnec *et al*. (2012) in their study of resistance genes for downy and powdery mildew in grapevine.

In the present study the coefficient of correlation of the marker TA194 with disease resistant gene (*FOC* locus), Factor 1 was highly significant at $P \geq 0.01$. Therefore, the SSR marker has shown a strong association with the presence of alleles for resistance. The PCR amplification using TA194 for certain accessions scored multiple bands, reported earlier by Holton *et al*., 2002 in their studies. Therefore re-synthesis of valid molecular SSR markers is required with a single amplified locus. One of the reasons for the appearance of multiple bands is the presence of cryptic sites of the primer binding sites (Winter *et al*., 1999). The accessions 2273 (Resistant) and 3058 (Moderately resistant) did not show any sort of band during PCR amplification that may be due to mutation in primer binding site or absence of the locus (Datta and Lal, 2011), because these accessions were found to be resistant during field screening.

Evaluation and selection of superior genotypes using various scientific techniques for utilization of yield enhancement on the basis of performance stability is considered an important research study all over the world. For which the initial step is to control the devastating *Fusarium* wilt disease of the crop through MAS to develop disease resistant germplasm of cultivated chickpea in Pakistan. The present study revealed *Fusarium* wilt resistant germplasm along with its linkage to SSR markers at the molecular weight of 204bp that could confidently be used in a future chickpea improvement program. The

initial step is to control the devastating *Fusarium* wilt disease of the crop using disease resistant lines; therefore the use of the selected wilt resistant genotypes through SSR marker TA194 can provide an opportunity in marker assisted breeding for yield improvement of the crop.

CONCLUSIONS

The present study concluded that:

1. The average value of coefficient of variation, which is calculated as 44.8% and 56.8% for qualitative and economically important quantitative traits respectively confirmed the existence of diversity among chickpea indigenous and exotic accessions.
2. In correlation studies positive and significant correlation has been observed between 100 seed weight, grain yield, total biomass and harvest index shown that chickpea yield could be improved by considering the improvement of any one selected quantitative trait.
3. Comparative frequency distribution data for genotypes recorded distinct variation with less environmental error (tEE. 5%) for 100 seed weight indicating seed weight as a stable trait.
4. Based on genetic information obtained using SDS-PAGE, about 50% genetic diversity was estimated in in the present work.
5. RAPD and SSR are highly polymorphic markers and can complement the genetic information collected from morphometric. So, these markers could be used for exploring the genetic diversity and phylogenetic relationship among USA and Pakistani chickpea germplasm.
6. SSR markers scored 55% genetic diversity which is comparatively higher (%) than that reported from SDS-PAGE (50%) and RAPD (37%). Thus SSR markers have proved to be more authentic tool for measuring the genetic diversity in chickpea.
7. Seed weight and seed size are important growth parameters and have a direct relationship with each other because in chickpea, seeds of quality size ranged from medium (7.2mm) to large (8-9.9mm) irrespective of desi or kabuli type have given comparatively better and higher yield in the evaluated germplasm. Thus the use of molecular markers in linkage analysis of yield contributing quantitative traits may provide a better chance to isolate medium to large size chickpea seeds to improve the production rate of the crop.
8. Screening of chickpea local and exotic accessions against *Fusarium* wilt disease through field evaluation and marker assisted selection (MAS) generated quite comparable data and showed that the SSR marker TA194 can be linked to wilt resistance gene to identify

resistant lines in a relatively shorter period of time for further utilization to improve the yield of a crop.

9. Analysis of chickpea germplasm through morphometric, biochemical and molecular markers can provide a chance for Scientists to direct selection of promising lines rather than followed by conventional breeding methods.

FINDINGS

1. Selected the elite and high yielding genotypes.
2. Determined the correlation among quantitative traits.
3. Selected *Fusarium* wilt resistant chickpea accessions.
4. Selected SSR markers for MAS breeding strategies of chickpeas.
5. Estimated the level of genetic diversity in chickpea germplasm using. Morphometric and molecular markers based on protein and DNA.

FUTURE RECOMMENDATIONS

1. The morphometric, biochemical (SDS-PAGE) and molecular markers (RAPD, SSR) reported in the study, are helpful to assess the extent of genetic diversity among indigenous and exotic chickpea accessions and can be used to identify the unreported cultivars with desirable quantitative traits for improving chickpea yield and genomic resources. It is therefore, suggested that these markers which have greatly supported the information of each other could be useful for the characterization and grouping of germplasm on the basis of their origin and performance.
2. Seed weight and seed size have a positive significant correlation, therefore seeds of quality size ranging from medium to larger (7.2mm- 9.9mm) irrespective of desi or kabuli type can be used for higher yield of cultivated chickpea in Pakistan.
3. The accessions 3054, 3056, 3043, 2553, 2855 and 2235 were found to be highly resistant at both seedling and reproductive stage to pod maturity stage in field, greenhouse and PCR screening. Therefore, these cultivars can be directly use by the breeders for sowing following some precautionary measures to eliminate the chances of diseases.
4. To improve chickpea breeding, it is highly recommended to ensure the use of disease free accessions and their cultivation in late sowing period to control the appearance of *Fusarium* wilt disease in chickpea growing areas of Pakistan.
5. Based on the study, the accessions 3043 and 3054 have also been recommended to the breeders for their future use in multiplication to increase yield of the crop and to conserve genetically superior germplasm resources.

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APPENDICES

Preparation of buffers

Appendix 1: Protein extraction buffer

(0.05M Tris-HCL Ph 8, 0.2% SDS, 5M urea, 1% β-mercaptoethanol)	
Chemicals	Required weight/ volume
Tris	0.6057g
Sodium dodecyl sulphate (SDS)	0.2g
Urea	30.3g
Distilled water	7ml
Conc.HCL Ph 8	Adjust to Ph 8.0
2-mercaptoethanol (Sigma)	1ml
	Make total volume of 100 ml

Appendix 2: Electrode buffer solution

(0.05M Tris, 0.192 M Glycin, 0.125% SDS)	
Chemicals	Required weight/ volume
Tris	3g
Sodium dodecyl sulphate (SDS)	1.25g
Glycine+ distilled water	14.4g
Stored at room temperature	Make total volume of 100 ml

Appendix 3: Staining solution

Staining solution for SDS-PAGE Gel	
Chemicals	Required weight/ volume
Methanol	440 ml
Acetic acid	60 ml
Distilled water	500 ml
Coomassie brilliant blue (CBB) R 250	2.25 g
Stored at room temperature after stirring for 30 min.	

Appendix 4: De-Staining solution

De-staining solution for SDS-PAGE Gel	
Chemicals	Required weight/ volume
Methanol	200 ml
Acetic acid	50 ml
Distilled water	750 ml
Stored at room temperature after stirring for 30 min.	

Solutions for gel electrophoresis

Appendix 5: Solution A

(3M Tris-HCL pH8.8, 0.4% SDS)	
Chemicals	Required weight/ volume
Tris	3g
Sodium dodecyl sulphate (SDS)	0.4g
Distilled water+Conc. HCL to adjust	70 ml
Stored in refrigerator	pH8.8 with final volume 100ml

Appendix 6: Solution B

(0.493M Tris-HCL pH7, 0.4% SDS)	
Chemicals	Required weight /volume
Tris	5.980g
Sodium dodecyl sulphate (SDS)	0.4g
Distilled water+Conc. HCL to adjust	80 ml
Stored in refrigerator	pH7 with final volume 100ml

Appendix 7: Solution C

(30% Acrylamide, Bis-acrylamide-30:0.8)	
Chemicals	Required weight/ volume
Acrylamide (Sigma)	30g
Bis-acrylamide	0.8g
Distilled water	Make a volume of 100 ml

Appendix 8: Solutions for 1mm Thick gels

Separation gel	12%
Solution A	5 ml
Solution C	7.5 ml
APS	200 µl
Distilled water	7.5 ml
TEMED	15µ

Appendix 9: Staking gel

Staking gel	4.50%
Solution B	2.5 ml
Solution C	1.5 ml
APS	70 µl
Distilled water	5 ml
TEMED	17µl

TEMED should be added at the end and shake well to avoid bubbles

Appendix 10: SSR-PCR data using TA194

S.No	Accession No.	TA194	S.No	Accession No.	TA194	S.No	Accession No.	TA194
1	1898	1	24	2562	1	48	3033	0
2	1936	0	25	2595	1	49	3035	1
3	1995	1	26	2611	1	50	3037	1
4	1998	1	27	2616	0	51	3039	1
5	2023	1	28	2629	0	52	3040	0
6	2188	1	29	2650	0	53	3041	1
7	2234	0	30	2654	1	54	3042	1
8	2235	1	31	2819	1	55	3043	1
9	2236	1	32	2831	1	56	3044	0
10	2237	0	33	2855	1	57	3045	1
11	2272	1	34	2859	0	58	3046	1
12	2273	0	35	3011	1	59	3047	0
13	2278	0	36	3015	1	60	3054	1
14	2430	1	37	3016	0	61	3056	1
15	2441	1	38	3017	0	62	3057	1
16	2473	1	39	3020	1	63	3058	0
17	2497	0	40	3021	1	64	3059	1
18	2499	1	41	3022	0	65	3061	1
19	2531	1	42	3023	1	66	3062	0
20	2532	1	43	3024	1	67	3063	1
21	2544	0	44	3026	1	68	3064	0
22	2553	1	45	3027	0	69	3065	1
23	2558	1	46	3031	0	70	3066	1
			47	3032	1			

Appendix 11: SSR-PCR data of genes linked with yield traits

S.No	Accession No.	TA72	TA130	S.No	Accession No.	TA72	TA130
1	1898	1	0	36	3015	1	0
2	1936	1	0	37	3016	0	0
3	1995	1	0	38	3017	1	0
4	1998	1	1	39	3020	1	0
5	2023	1	1	40	3021	1	1
6	2188	1	0	41	3022	1	0
7	2234	0	1	42	3023	1	1
8	2235	1	1	43	3024	0	1
9	2236	0	0	44	3026	1	0
10	2237	0	1	45	3027	1	0
11	2272	1	0	46	3031	1	0
12	2273	0	1	47	3032	1	1
13	2278	1	0	48	3033	1	0
14	2430	1	0	49	3035	1	0
15	2441	0	0	50	3037	1	0
16	2473	1	0	51	3039	1	0
17	2497	1	0	52	3040	1	1
18	2499	0	0	53	3041	1	0
19	2531	1	0	54	3042	1	1
20	2532	1	0	55	3043	1	0
21	2544	1	0	56	3044	0	0
22	2553	1	0	57	3045	1	0
23	2558	1	0	58	3046	0	0
24	2562	0	1	59	3047	1	0
25	2595	0	1	60	3054	1	0
26	2611	0	0	61	3056	1	0
27	2616	0	0	62	3057	1	0
28	2629	0	0	63	3058	1	0
29	2650	0	0	64	3059	0	1
30	2654	0	0	65	3061	0	1
31	2819	1	0	66	3062	1	0
32	2831	0	0	67	3063	1	1
33	2855	1	0	68	3064	1	0
34	2859	1	0	69	3065	1	0
35	3011	1	1	70	3066	1	0

Appendix 12: Field screening data of chickpea 70 accessions against *Fusarium* wilt disease

Accessions distributed with reference to disease response at seedling stage	No. of acc. Contributed	1-9 rating scale score	Disease response
1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2562, 2595, 2611, 3037, 3039, 3043, 3054, 3056, 2819, 2831, 3059, 2855.	20	1	Highly resistant
2272, 2273, 2473, 2499, 2531, 2558, 2654, 3011, 2532, 3020, 3021, 3023, 3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063.	20	3	Resistant
1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061.	09	5	Moderatel y resistant
3027, 3031, 3033, 3040, 3044, 3047, 2629, 2650, 2859, 3062, 3064, 2544, 2234, 1936, 2237, 2278, 2497, 3022, 3017, 3016, 2616.	21	7	Susceptibl e

Appendix 13: Field screening data of chickpea 70 accessions against *Fusarium* wilt disease

Accessions distributed with reference to disease response at Reproductive stage	No. of acc. Contributed	1-9 rating scale score	Disease response
1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 2611, 3043, 3054, 3059, 2855.	14	1	Highly resistant
2272, 2273, 2473, 2531, 2654, 3011, 2532, 3020, 3021, 3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063.	17	3	Resistant
1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061,	09	5	Moderately resistant
3027, 3031, 3033, 3040, 3044, 3047, 2629, 2650, 2859, 3062, 3064, 2544, 2234, 1936, 2237, 2278, 2497, 3022, 3017, 3016, 2616, 3023, 2499, 2558, 3039, 3056, 2831, 2819, 3037, 2562.	30	7	Susceptible

Appendix 14: Greenhouse disease screening data of chickpea 70 accessions against *Fusarium* wilt disease

Accessions distributed with reference to disease response at seedling stage	No. of acc. Contributed	1-9 rating scale score	Disease response
1898, 2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 2611, 3037, 3039, 3043, 3054, 3056, 2819, 3059, 2855.	18	1	Highly resistant
2272, 2273, 2473, 2499, 2531, 2558, 2654, 3011, 2532, 3020, 3021, 3023, 3035, 3041, 3045, 3046, 3057, 3065, 3066, 3063, 1995, 1998, 3015, 3032, 3042, 3026, 3024, 3058, 3061, 3040, 2831, 2562.	32	3	Resistant
3047, 3022, 1936, 2859, 3062, 3064, 2544, 3017, 3016, 2616, 2237, 3031, 3033, 3044	14	5	Moderately resistant
3027, 2629, 2650, 2234, 2278, 2497	06	7	Susceptible

Appendix 15: Greenhouse disease screening data of chickpea 70 accessions against *Fusarium* wilt disease

Accessions distributed with reference to disease response at reproductive stage	No. of acc. Contributed	1-9 rating scale score	Disease response
2023, 2188, 2235, 2236, 2430, 2441, 2553, 2595, 3039, 3043, 3054, 3056, 2819, 3059, 2855.	15	1	Highly resistant
2272, 2273, 2473, 2499, , 2558, 2654, 3011, 2532, 3020, 3021, 3023, 3035, 3045, 3046, 3057, 3065, 3066, 3063, 1995, , 3015, 3032, 3042, , 3024, 3058, 3061, 3040, ,.	26	3	Resistant
3047, 3022, 1936, 2859, 3062, 3064, 2544, 3017, 3016, 2616, , 3033, 3044	12	5	Moderately resistant
1898, 1998, 2611, 3027, 2629, 2650, 2234, 2278, 2497, 3037, 2562, 2531, 3026, 2831, 3041, 2237, 3031.	17	7	Susceptible

Appendix 16: SDS-PAGE analysis of chickpea 70 accessions

S#	Acc.#	C. of origin	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16
1	1898	Pakistan	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1
2	1936	Pakistan	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1
3	1995	Pakistan	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1
4	1998	Pakistan	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1
5	2023	Pakistan	0	1	1	0	1	0	1	1	0	0	1	1	1	0	0	1
6	2188	Pakistan	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1
7	2234	Pakistan	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1
8	2235	Pakistan	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1
9	2236	Pakistan	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0
10	2237	Pakistan	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
11	2272	Pakistan	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
12	2273	Pakistan	0	0	0	0	0	1	1	1	0	0	1	1	1	1	1	1
13	2278	Pakistan	0	0	1	1	1	0	1	0	1	1	0	0	1	1	1	1
14	2430	Pakistan	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1
15	2441	Pakistan	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	2473	Pakistan	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	2497	Pakistan	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
18	2499	Pakistan	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1
19	2531	Pakistan	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
20	2532	Pakistan	0	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0
21	2544	Pakistan	0	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0
22	2553	Pakistan	0	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0
23	2558	Pakistan	0	1	1	1	0	1	0	0	1	0	0	0	1	1	0	0
24	2562	Pakistan	1	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0
25	2595	USA	1	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0
26	2611	USA	1	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1
27	2616	USA	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0
28	2629	USA	1	1	1	1	1	1	0	1	1	0	0	1	0	1	0	0
29	2650	USA	1	1	1	1	1	1	0	0	1	0	0	0	0	1	0	0
30	2654	USA	1	1	0	1	1	1	1	1	1	0	1	1	0	0	1	0
31	2819	USA	1	1	0	0	1	1	1	1	1	0	1	1	0	0	1	0
32	2831	USA	1	1	0	0	1	1	1	1	1	0	1	1	0	0	1	0
33	2855	USA	1	1	0	0	1	1	1	1	1	0	1	1	0	0	1	0
34	2859	USA	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
35	3011	USA	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1
36	3015	USA	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
37	3016	USA	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0

38	3017	USA	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0
39	3020	USA	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
40	3021	USA	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1
41	3022	USA	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1
42	3023	USA	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
43	3024	USA	0	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1
44	3026	USA	0	1	1	1	0	0	1	0	0	1	1	1	0	1	0	1
45	3027	USA	1	1	1	0	0	0	0	0	0	1	1	1	1	0	1	1
46	3031	USA	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1
47	3032	USA	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
48	3033	USA	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
49	3035	USA	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1
50	3037	USA	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1
51	3039	USA	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1
52	3040	USA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
53	3041	USA	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	1
54	3042	USA	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1
55	3043	USA	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1
56	3044	USA	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1
57	3045	USA	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	1
58	3046	USA	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
59	3047	USA	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
60	3054	USA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
61	3056	USA	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1
62	3057	USA	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0
63	3058	USA	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
64	3059	USA	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
65	3061	USA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
66	3062	USA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
67	3063	USA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
68	3064	USA	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1
69	3065	USA	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1
70	3066	USA	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1

Appendix 17: Morphological evaluation of four quantitative traits during the year 2008-2009

		Field Data-2008-2009						Field Data-2008-2009			
S/No.	Acc.#	100/ seed weight	Grain yied	Total Biom	Harvest Index	S/No.	Acc.#	100/ seed weight	Grain yied	Total Biom	Harvest Index
1	1898	21.18	105.27	250	42.108	36	3015	26	86.03	500	17.206
2	1936	16.56	101.72	350	29.06	37	3016	14.37	113.2	470	24.08
3	1995	17.99	88.43	350	25.26	38	3017	23.05	51.37	350	14.67
4	1998	28.62	86.52	400	21.63	39	3020	21.71	100.59	500	20.11
5	2023	31.09	119.08	600	19.84	40	3021	34.56	121.18	500	24.23
6	2188	13.71	60.75	200	30.37	41	3022	29.24	101.64	850	11.95
7	2234	21.67	217.82	700	31.11	42	3023	30.08	66.36	400	16.59
8	2235	12.57	21.7	100	21.7	43	3024	25.8	150	680	22.05
9	2236	19.42	175.4	550	31.89	44	3026	44.25	144.08	500	28.81
10	2237	12.36	6.9	200	3.45	45	3027	57.76	38.05	100	38.05
11	2272	19.95	48.78	200	24.39	46	3031	15.85	50.45	250	20.18
12	2273	32.01	27.52	200	13.76	47	3032	23.68	88.06	450	19.56
13	2278	16.78	18.579	350	5.308	48	3033	17.03	46.18	300	15.39
14	2430	16.31	5.45	50	10.9	49	3035	23.87	109.5	600	18.25
15	2441	24.9	1.2	150	0.8	50	3037	56.87	1.97	900	0.2188
16	2473	19.95	46.18	300	15.39	51	3039	55.18	169.48	600	28.24
17	2497	21.18	105.27	250	42.108	52	3040	40.08	79.93	850	9.403
18	2499	18.34	52.38	100	52.38	53	3041	25.3	121.87	600	20.31
19	2531	18.57	27.68	200	13.84	54	3042	28.2	168.32	650	25.89
20	2532	19.01	45.6	250	18.24	55	3043	48.3	169.7	900	18.85
21	2544	16.81	57.5	150	38.33	56	3044	35.47	35.31	550	6.42
22	2553	18.05	82.03	350	23.43	57	3045	32.71	27.52	450	6.11
23	2558	19.02	34.8	250	13.92	58	3046	19.67	142.8	300	47.6
24	2562	31.4	60.14	300	20.04	59	3047	48.53	145.97	550	26.54
25	2595	23.68	34.34	350	9.811	60	3054	51.39	287.56	1150	25.00
26	2611	12.82	6.9	100	6.9	61	3056	52.04	129.5	600	21.58
27	2616	27.54	55.5	140	39.64	62	3057	25.11	50.04	500	10.008
28	2629	20.24	120.58	300	40.19	63	3058	38.7	150.6	350	43.02
29	2650	23.27	61.78	400	15.44	64	3059	31.69	171.11	900	19.01
30	2654	34.74	150.3	850	17.68	65	3061	30	123.48	500	24.69
31	2819	20.93	159.18	350	45.48	66	3062	29.63	72.43	500	14.48
32	2831	25.25	77.66	550	14.12	67	3063	32.72	196.91	900	21.87
33	2855	26.53	66.86	350	19.10	68	3064	29.7	104.35	750	13.91
34	2859	22.69	70.8	400	17.7	69	3065	33.56	121.34	600	20.22
35	3011	29.46	80.92	450	17.98	70	3066	31.83	95.6	400	23.9

Appendix 18: Morphological evaluation of four quantitative traits during the year 2009-10

		Field Data-2009-2010						Field Data-2009-2010			
S/No.	Acc.#	100/ seed weight	Grain yield	Total Biom	Harvest Index	S/No.	Acc.#	100/ seed weight	Grain yield	Total Biom	Harvest Index
1	1898	23.1	115.01	138	83.34	36	3015	30.15	95.5	710	13.45
2	1936	38.52	60.15	151	39.83	37	3016	17.72	105.11	600	17.51
3	1995	20.11	60.2	185	32.54	38	3017	22.15	45.22	250	18.08
4	1998	24.12	120.11	246	48.82	39	3020	18.66	86.62	470	18.42
5	2023	40.23	70.12	385	18.21	40	3021	30.17	130.6	400	32.65
6	2188	38.31	80.51	190	42.37	41	3022	31.22	99.61	938	10.61
7	2234	30.15	41.12	439	9.366	42	3023	29.65	73.1	513	14.24
8	2235	18.12	36.1	167	21.61	43	3024	26.8	213.33	745	28.63
9	2236	19.52	80.82	405	19.95	44	3026	35.15	181.23	490	36.98
10	2237	12.92	21.11	219	9.639	45	3027	21.71	30.03	115	26.11
11	2272	23.95	60.12	280	21.47	46	3031	17.38	63.14	310	20.36
12	2273	18.12	19.23	111	17.32	47	3032	16.12	66.11	290	22.79
13	2278	19.41	22.41	138	16.23	48	3033	20.11	46.12	312	14.78
14	2430	18.31	55.73	381	14.62	49	3035	15.98	112.63	670	16.81
15	2441	20.12	41.33	115	35.93	50	3037	56.31	63.64	702	9.065
16	2473	19.11	29.12	353	8.249	51	3039	41.19	172.11	710	24.24
17	2497	26.18	115.2	290	39.72	52	3040	52.75	85.12	925	9.202
18	2499	8.53	10.11	101	10.00	53	3041	28.2	140.33	680	20.63
19	2531	22.1	40.91	114	35.88	54	3042	38.1	175.1	700	25.01
20	2532	23.1	42.21	302	13.97	55	3043	45.28	172.5	900	19.16
21	2544	17.18	59.12	131	45.12	56	3044	35.02	35.22	670	5.256
22	2553	19.12	55.1	394	13.98	57	3045	20.41	21.1	430	4.906
23	2558	20.1	33.71	173	19.48	58	3046	19.6	99.56	350	28.44
24	2562	29.61	71.23	427	16.68	59	3047	18.23	112.32	650	17.28
25	2595	18.68	40.23	500	8.046	60	3054	59.2	316.71	1322	23.95
26	2611	11.1	7.1	120	5.916	61	3056	64.51	113.66	650	17.48
27	2616	23.12	56.41	148	38.11	62	3057	25	33.71	525	6.420
28	2629	17.22	110.21	250	44.08	63	3058	30.7	112.61	400	28.15
29	2650	25.01	65.73	450	14.60	64	3059	55.26	203.12	1256	16.17
30	2654	29.71	143.11	400	35.77	65	3061	19.78	98.65	490	20.13
31	2819	26.55	143.1	400	35.77	66	3062	20.03	70.35	425	16.55
32	2831	25.15	78.41	630	12.44	67	3063	28.62	112.6	945	11.91
33	2855	20.33	63.64	361	17.62	68	3064	29.7	112.67	860	13.10
34	2859	30.11	71.94	443	16.23	69	3065	39.56	102.63	615	16.68
35	3011	16.45	69.31	413	16.78	70	3066	32.18	75.12	380	19.76

Appendix 19: Morphological evaluation of four quantitative traits during the year 2010-11

		Field Data-2010-2011						Field Data-2010-2011			
S/No.	Acc.#	100/ seed weight	Grain yield	Total Biom	Harvest Index	S/No.	Acc.#	100/ seed weight	Grain yield	Total Biom	Harvest Index
1	1898	25.6	101.8	192	53.02	36	3015	34.1	13.4	680	1.97
2	1936	12.9	99.72	266	37.48	37	3016	14.37	22.2	600	3.7
3	1995	19.32	89.55	401	22.33	38	3017	40.12	15.1	221	6.83
4	1998	22.1	76.44	399	19.16	39	3020	53	84.7	612	13.84
5	2023	45.4	120	450	26.67	40	3021	30.6	155.9	466	33.45
6	2188	10.22	45.12	203	22.23	41	3022	28.99	18.5	925	2
7	2234	29	212.2	589	36.03	42	3023	35.45	36.3	504	7.2
8	2235	16.21	23.6	145	16.27	43	3024	16.21	141.9	345	41.13
9	2236	23.12	133.7	510	26.21	44	3026	33.5	112.6	250	45.04
10	2237	16.33	23.7	200	11.85	45	3027	33.5	22.5	99	22.72
11	2272	25.78	24.9	188	12.34	46	3031	55	13.9	366	3.79
12	2273	39.65	26.9	99	27.17	47	3032	24.66	20.9	190	11
13	2278	16.89	21.9	277	7.9	48	3033	22.54	20.4	219	9.31
14	2430	18.31	34.7	101	34.36	49	3035	16.12	98.6	450	21.91
15	2441	28.66	14.05	90	15.61	50	3037	56.8	20.9	890	2.35
16	2473	15.11	17.8	270	6.59	51	3039	25	228.9	650	35.07
17	2497	11.8	114.4	250	45.76	52	3040	39.99	19.4	1120	1.73
18	2499	27.55	22.3	87	25.63	53	3041	20	128.3	544	23.58
19	2531	23.54	13.4	322	4.16	54	3042	28.21	200	476	42.02
20	2532	23.1	16.7	312	5.35	55	3043	45	192.9	850	22.69
21	2544	15.4	16.8	133	12.63	56	3044	32.11	21.4	455	4.7
22	2553	22.7	99.8	405	24.64	57	3045	11.22	14.3	450	3.17
23	2558	30	16.8	200	8.4	58	3046	16.3	99	300	33
24	2562	29.99	18.8	250	7.52	59	3047	26	110.2	619	17.8
25	2595	31	23.1	623	3.7	60	3054	48.9	226.3	900	25.11
26	2611	19.33	15.3	90	17	61	3056	55	102.6	507	20.23
27	2616	20.12	20.1	109	18.44	62	3057	18.72	28.2	311	9.06
28	2629	18.12	117.8	289	40.76	63	3058	28.2	112.5	350	32.14
29	2650	18.11	10.5	508	2.066	64	3059	19.6	188.8	1422	13.27
30	2654	29	133.4	750	17.79	65	3061	38	99.9	460	21.72
31	2819	29.23	169.9	444	38.26	66	3062	25.1	28.8	450	6.4
32	2831	26.76	20.1	723	2.78	67	3063	50	201.8	1100	18.34
33	2855	33.5	19.1	300	6.37	68	3064	32.7	108.9	250	43.56
34	2859	26	12.3	267	4.1	69	3065	45	211.3	600	35.22
35	3011	30.96	25.3	413	6.12	70	3066	18.5	21.3	250	8.52

Appendix 20: Data obtained by polymerase chain reaction using OPA09 primer

Code No.	Accession No.	DNA bands or alleles No.						Code No.	Accession No.	DNA bands or alleles No.					
		1	2	3	4	5	6			1	2	3	4	5	6
1	1898	0	0	0	0	0	0	36	3015	0	0	0	0	0	0
2	1936	0	1	0	0	0	0	37	3016	0	0	1	0	1	0
3	1995	0	0	0	0	0	1	38	3017	0	0	0	0	0	0
4	1998	0	0	1	0	0	0	39	3020	0	0	1	0	1	0
5	2023	0	0	0	0	0	0	40	3021	0	0	0	0	0	0
6	2188	0	0	0	0	0	0	41	3022	0	0	0	0	0	0
7	2234	0	1	0	0	0	0	42	3023	0	0	0	0	0	0
8	2235	0	1	0	0	0	0	43	3024	1	0	0	0	0	0
9	2236	0	0	0	0	0	0	44	3026	0	0	0	0	0	1
10	2237	0	0	0	0	0	0	45	3027	0	0	1	0	1	0
11	2272	0	0	0	0	1	0	46	3031	0	0	0	0	0	0
12	2273	0	0	1	0	1	0	47	3032	0	0	1	0	1	0
13	2278	1	0	0	0	0	0	48	3033	0	0	1	0	1	0
14	2430	0	0	0	0	1	0	49	3035	0	0	0	0	0	0
15	2441	0	0	0	0	1	0	50	3037	0	0	0	0	0	1
16	2473	0	0	0	0	0	0	51	3039	0	0	0	0	0	0
17	2497	0	1	0	1	0	0	52	3040	0	0	1	0	0	0
18	2499	0	0	0	0	0	0	53	3041	0	0	1	0	1	0
19	2531	0	0	0	1	0	0	54	3042	0	0	1	0	1	0
20	2532	0	0	1	0	1	0	55	3043	0	0	1	0	1	0
21	2544	0	0	1	0	1	0	56	3044	1	0	0	0	0	0
22	2553	0	0	0	0	0	0	57	3045	0	0	1	0	1	0
23	2558	0	0	0	0	1	0	58	3046	0	0	1	0	1	0
24	2562	0	0	0	0	0	0	59	3047	0	0	0	0	0	0
25	2595	0	0	0	0	0	0	60	3054	0	0	1	0	1	0
26	2611	0	0	0	0	0	0	61	3056	0	0	1	0	1	0
27	2616	0	0	1	0	1	0	62	3057	0	0	1	0	1	0
28	2629	0	0	0	0	0	0	63	3058	1	0	0	0	0	0
29	2650	0	0	0	0	0	0	64	3059	0	0	1	0	1	0
30	2654	0	0	1	0	1	0	65	3061	0	0	1	0	1	0
31	2819	0	0	0	0	0	0	66	3062	0	0	0	0	1	0
32	2831	0	0	1	0	1	0	67	3063	0	0	0	0	0	0
33	2855	0	0	0	0	0	0	68	3064	0	0	0	0	0	1
34	2859	0	0	0	0	0	0	69	3065	0	0	1	0	1	0
35	3011	0	0	1	0	1	0	70	3066	0	0	0	0	0	0

Appendix 21: Data obtained by polymerase chain reaction using OPA04 primer

Code No.	Accession No.	DNA bands or alleles No.								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	1898	0	0	0	0	0	0	0	0	0
2	1936	1	1	0	0	0	1	1	0	0
3	1995	0	0	0	0	1	0	1	0	0
4	1998	0	0	0	0	0	0	0	0	0
5	2023	0	0	0	0	1	0	1	0	0
6	2188	0	0	0	0	0	0	1	0	0
7	2234	0	0	0	0	0	0	0	0	0
8	2235	0	0	0	0	0	0	1	0	0
9	2236	0	0	0	0	0	0	1	0	0
10	2237	0	0	0	0	0	0	1	0	0
11	2272	0	0	0	0	0	0	0	0	0
12	2273	0	0	0	0	0	0	0	0	0
13	2278	0	0	0	0	0	0	1	0	0
14	2430	0	0	0	0	0	0	0	0	0
15	2441	0	0	0	0	0	0	1	0	0
16	2473	0	0	0	0	0	0	0	0	0
17	2497	0	0	1	1	0	0	0	0	0
18	2499	0	0	0	0	0	0	0	0	0
19	2531	0	0	0	0	0	0	0	0	0
20	2532	0	0	0	0	0	0	0	0	0
21	2544	0	0	0	0	0	0	0	0	0
22	2553	0	0	1	0	1	0	0	0	0
23	2558	0	0	0	0	1	0	0	0	0
24	2562	0	0	0	0	0	0	0	0	0
25	2595	0	0	1	0	0	0	0	0	0
26	2611	0	0	0	0	0	0	0	0	0
27	2616	0	0	0	0	0	0	1	0	0
28	2629	0	0	0	1	1	0	0	0	0
29	2650	0	0	0	0	0	0	1	0	0
30	2654	0	0	0	0	0	0	1	0	0
31	2819	0	0	0	0	0	0	0	0	0
32	2831	0	0	0	0	0	0	1	0	0
33	2855	0	0	0	0	0	0	0	0	0
34	2859	0	0	0	0	0	0	0	0	0
35	3011	0	0	0	0	0	0	0	0	0
36	3015	0	0	0	0	1	0	0	1	0
37	3016	0	0	0	0	0	0	0	0	0
38	3017	0	0	0	0	1	0	0	1	0
39	3020	0	0	0	0	0	0	1	0	0
40	3021	0	0	0	0	0	0	0	1	1
41	3022	0	0	0	0	0	0	0	0	0
42	3023	0	0	0	0	1	0	0	1	0
43	3024	0	0	0	0	0	0	0	0	0
44	3026	0	0	0	0	0	1	1	0	0
45	3027	0	0	0	0	0	0	0	0	0
46	3031	0	0	0	0	0	0	0	0	0
47	3032	0	0	0	0	0	0	0	0	0
48	3033	0	0	0	0	0	0	0	0	0
49	3035	0	0	0	0	0	1	0	1	0

50	3037	0	0	0	0	0	0	0	0	0
51	3039	0	0	0	0	0	0	0	1	0
52	3040	0	0	0	0	0	0	0	0	0
53	3041	0	0	0	0	1	0	0	1	0
54	3042	0	0	0	0	0	1	0	1	0
55	3043	0	0	0	0	0	0	0	0	0
56	3044	0	0	0	0	0	0	0	1	0
57	3045	0	0	0	0	0	0	0	0	0
58	3046	0	0	0	0	0	0	0	0	0
59	3047	0	0	0	0	0	1	0	1	0
60	3054	0	0	0	0	0	0	0	1	0
61	3056	0	0	0	0	0	0	1	0	0
62	3057	0	0	0	0	0	0	0	1	0
63	3058	0	0	0	0	0	0	0	1	0
64	3059	0	0	0	0	0	0	0	0	0
65	3061	0	0	0	0	0	1	0	1	0
66	3062	0	0	0	0	0	0	0	0	0
67	3063	0	0	0	0	0	0	0	1	0
68	3064	0	0	0	0	0	1	0	1	0
69	3065	0	0	0	0	0	0	1	0	0
70	3066	0	0	0	0	0	0	1	0	0

Appendix 22: Data obtained by polymerase chain reaction using UBC733b primer

Code No.	Accession No.	DNA bands or alleles No.																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1898	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0
2	1936	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
3	1995	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
4	1998	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
5	2023	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0
6	2188	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0
7	2234	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0
8	2235	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
9	2236	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0
10	2237	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
11	2272	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
12	2273	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
13	2278	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0
14	2430	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0
15	2441	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
16	2473	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
17	2497	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
18	2499	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	2531	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	2532	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
21	2544	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	2553	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0
23	2558	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
24	2562	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0
25	2595	0	0	0	1	0	0	1	0	1	1	0	1	0	0	0	0	0
26	2611	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
27	2616	1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0
28	2629	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
29	2650	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0
30	2654	0	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	0
31	2819	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
32	2831	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0
33	2855	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
34	2859	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
35	3011	0	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0
36	3015	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	3016	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	3017	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	3020	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1
40	3021	0	0	1	0	1	0	1	0	1	1	0	0	0	1	1	0	1
41	3022	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0
42	3023	0	0	1	0	0	0	1	1	0	0	0	0	1	0	0	1	1
43	3024	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1
44	3026	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1
45	3027	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
46	3031	1	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1
47	3032	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
48	3033	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
49	3035	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0

50	3037	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	3039	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
52	3040	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0
53	3041	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
54	3042	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	1	0
55	3043	0	0	1	0	1	0	0	1	1	0	0	0	1	0	1	0	0
56	3044	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
57	3045	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	3046	0	0	1	1	0	0	1	1	0	0	0	0	0	1	0	0	0
59	3047	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0
60	3054	1	0	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0
61	3056	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
62	3057	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
63	3058	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64	3059	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	0	0
65	3061	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0
66	3062	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
67	3063	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
68	3064	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
69	3065	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
70	3066	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Appendix 23: Data obtained by polymerase chain reaction using UBC181 primer

Code No.	Accession No.	DNA bands or alleles No.																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1898	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	1	0
2	1936	0	0	1	1	0	1	1	1	0	0	1	1	0	1	1	0	0
3	1995	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	0	0
4	1998	0	0	0	0	1	1	0	0	0	1	0	1	1	1	0	0	0
5	2023	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	1	0
6	2188	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0
7	2234	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
8	2235	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
9	2236	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0
10	2237	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	2272	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0
12	2273	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	2278	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0
14	2430	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0
15	2441	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	2473	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0
17	2497	1	0	1	0	0	0	1	1	0	0	1	1	0	1	0	0	0
18	2499	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	2531	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	2532	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
21	2544	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0
22	2553	1	0	1	0	0	0	1	1	1	0	1	1	1	0	0	0	0
23	2558	1	0	1	0	0	0	1	1	1	0	1	0	1	0	0	0	0
24	2562	0	0	0	0	1	1	1	1	1	0	1	0	0	1	0	0	0
25	2595	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	0	0
26	2611	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0
27	2616	0	1	0	0	1	1	1	0	0	1	0	1	1	0	0	0	0
28	2629	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	0
29	2650	0	0	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0
30	2654	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0
31	2819	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0
32	2831	0	0	0	1	1	1	1	1	1	1	0	1	0	1	0	0	0
33	2855	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	1
34	2859	0	0	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0
35	3011	0	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1
36	3015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	3016	1	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0
38	3017	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
39	3020	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
40	3021	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1
41	3022	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
42	3023	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
43	3024	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0
44	3026	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
45	3027	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
46	3031	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0
47	3032	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0
48	3033	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1
49	3035	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0

50	3037	1	0	0	0	1	1	0	0	1	0	0	1	0	0	1	1	1
51	3039	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1
52	3040	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
53	3041	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	1	0
54	3042	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
55	3043	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
56	3044	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
57	3045	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
58	3046	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
59	3047	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
60	3054	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
61	3056	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
62	3057	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
63	3058	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64	3059	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
65	3061	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
66	3062	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
67	3063	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0
68	3064	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
69	3065	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0
70	3066	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0

Appendix 24: Data obtained by polymerase chain reaction using OPG13 primer

Code No.	Accession No.	DNA bands or alleles No.															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1898	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0
2	1936	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
3	1995	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0
4	1998	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
5	2023	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0
6	2188	0	0	0	0	1	1	1	0	1	0	0	1	1	0	0	0
7	2234	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
8	2235	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
9	2236	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0
10	2237	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0
11	2272	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0
12	2273	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
13	2278	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
14	2430	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
15	2441	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
16	2473	0	0	0	1	0	1	0	1	0	0	0	1	0	0	1	0
17	2497	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0
18	2499	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0
19	2531	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0
20	2532	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
21	2544	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
22	2553	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0
23	2558	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0
24	2562	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
25	2595	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
26	2611	0	0	0	1	0	1	0	1	0	1	0	1	0	0	0	0
27	2616	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
28	2629	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
29	2650	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
30	2654	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
31	2819	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
32	2831	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
33	2855	0	0	1	0	1	0	0	0	1	0	0	1	1	0	0	0
34	2859	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
35	3011	1	1	1	0	0	0	1	0	1	1	0	0	1	0	0	0
36	3015	0	1	1	0	1	1	0	1	1	0	1	1	0	0	0	0
37	3016	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0
38	3017	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0
39	3020	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
40	3021	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
41	3022	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
42	3023	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
43	3024	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
44	3026	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0
45	3027	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
46	3031	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0
47	3032	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
48	3033	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0
49	3035	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

50	3037	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0
51	3039	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0
52	3040	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0
53	3041	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0
54	3042	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0
55	3043	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	3044	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
57	3045	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	3046	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0
59	3047	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
60	3054	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
61	3056	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
62	3057	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0
63	3058	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
64	3059	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
65	3061	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
66	3062	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
67	3063	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
68	3064	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0
69	3065	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1
70	3066	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0

Appendix 25: SSR markers PCR data for determination of genetic diversity in 70 chickpeas

S/No.	Acc. No.	CaSTMS2	CaST MS15	CAT MS21	TA 71	TA 72	TA 130	TA 194	TA 22	TA 200	TA 46	TA 135	TR 1	TR 7	TR 29	TR 31
1	1898	0	1	1	1	1	0	1	1	1	1	1	0	1	1	1
2	1936	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1
3	1995	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1
4	1998	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1
5	2023	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	2188	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1
7	2234	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1
8	2235	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1
9	2236	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1
10	2237	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1
11	2272	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
12	2273	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1
13	2278	1	1	0	1	1	0	0	0	1	1	1	1	1	1	1
14	2430	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0
15	2441	0	0	1	1	0	0	1	1	1	1	0	1	1	1	1
16	2473	1	0	0	1	1	0	1	1	1	0	0	1	1	1	1
17	2497	1	1	0	1	1	0	0	1	1	0	1	1	1	1	0
18	2499	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1
19	2531	0	0	0	1	1	0	1	1	1	0	1	1	1	0	1
20	2532	0	1	1	0	1	0	1	1	0	1	1	1	1	1	0
21	2544	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0
22	2553	0	0	1	1	1	0	1	1	0	1	0	1	1	1	0
23	2558	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1
24	2562	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1
25	2595	1	0	0	1	0	1	1	1	1	0	1	1	1	1	1
26	2611	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0
27	2616	0	1	1	1	0	0	0	1	1	1	1	1	1	0	1
28	2629	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1
29	2650	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1
30	2654	0	0	1	1	0	0	1	1	1	1	1	0	1	1	0
31	2819	0	0	1	1	1	0	1	1	0	1	1	1	1	1	0
32	2831	0	1	0	1	0	0	1	1	0	1	1	1	1	1	0
33	2855	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1
34	2859	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0
35	3011	0	1	0	1	1	1	1	0	0	1	0	0	0	1	0
36	3015	1	0	1	1	1	0	1	0	0	1	1	0	1	1	0
37	3016	1	1	1	1	0	0	0	0	1	0	1	1	1	0	1
38	3017	1	1	1	1	1	0	0	1	0	1	1	1	1	0	1
39	3020	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1
40	3021	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
41	3022	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1
42	3023	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1
43	3024	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1
44	3026	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1
45	3027	1	1	0	1	1	0	0	0	1	0	1	1	1	1	1
46	3031	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1
47	3032	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1
48	3033	1	1	1	1	1	0	0	1	1	0	1	0	0	1	1
49	3035	1	1	1	1	1	0	1	0	1	0	1	0	0	1	1
50	3037	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0

51	3039	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0
52	3040	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1
53	3041	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1
54	3042	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1
55	3043	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0
56	3044	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1
57	3045	0	0	1	1	1	0	1	1	1	1	1	0	1	1	1
58	3046	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1
59	3047	1	0	1	1	1	0	0	1	1	1	0	0	1	1	1
60	3054	0	0	1	1	1	0	1	1	1	1	1	0	1	1	1
61	3056	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1
62	3057	0	1	1	1	1	0	1	1	1	1	1	0	1	1	1
63	3058	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1
64	3059	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1
65	3061	0	1	0	1	0	1	1	1	1	1	1	0	0	1	1
66	3062	0	0	0	1	1	0	0	1	1	1	1	0	0	1	1
67	3063	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
68	3064	1	0	0	1	1	0	0	1	1	1	0	1	1	1	1
69	3065	0	0	0	1	1	0	1	1	1	1	1	1	0	1	1
70	3066	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1